João Pedro Nunes Paulo da Silva Martins
Licenciado em Biologia Celular e Molecular

Nuclear movement to the periphery of skeletal muscle cells

Dissertação para obtenção do Grau de Mestre em Genética Molecular e Biomedicina

Orientador: Edgar Rodrigues Almeida Gomes, PhD, Instituto de Medicina Molecular

Júri:

Presidente: Prof. Doutora Margarida Casal Ribeiro Castro Caldas Braga
Arguente: Prof. Doutor Sérgio Jerónimo Rodrigues Dias

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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LOMBADA

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Agradecimentos

Muito obrigado Edgar pela oportunidade que me deste para trabalhar no laboratório, foi um privilégio. Obrigado pela disponibilidade e pelas palavras de apoio e incentivo ao longo do ano. Mas principalmente obrigado por me teres proporcionado as ferramentas necessárias para que eu me tornasse um cientista melhor e uma pessoa melhor.

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Um grande obrigado a toda a minha família pelo apoio que me têm dado ao longo dos anos. Em especial, aos meus pais e à minha irmã que tanta paciência e compreensão demonstraram ao longo dos anos que me permitiram chegar onde cheguei.

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Resumo

Os movimentos do núcleo das células são conduzidos por forças polarizadas exercidas por proteínas motoras e pelo citoesqueleto, sendo importantes para uma multiplicidade de funções celulares. Durante o desenvolvimento e regeneração do músculo esquelético os núcleos movem-se do centro para a periferia da miofibra. Este movimento tem início com a formação de uma dobra nuclear entre miofibrilas, que aumenta gradualmente de tamanho originando um hérnia nuclear. Durante o movimento para a periferia, o núcleo sofre deformações dramáticas devido à pressão das miofibrilas, até atingir a periferia da miofibra. O posicionamento dos núcleos na periferia das miofibras é crucial para uma funções muscular ideal, visto que núcleos localizados no centro de miofibras estão relacionados com várias patologias musculares. Com este trabalho conseguimos demonstrar que o mecanismo de movimento nuclear para a periferia depende de alterações locais da rigidez nuclear regulada por Lamina A/C. Descobrimos também que este movimento é controlado por reticulação de miofibrilas dependente de Desmina que as aproxima num processo semelhante a um fecho éclair. Por outro lado a Plectina, Arpc5L e γ actina são responsáveis pela organização desta rede de Desmina nas linhas-Z. As Nesprinas são componentes principais do complexo LINC e também estão envolvidas neste mecanismo. Por fim, a depleção de Nesprina1 originou uma redução considerável de núcleos à periferia o que sugere que estas proteínas também estão envolvidas no movimento nuclear para a periferia, possivelmente através de mecanotransdução

Palavras-chave: Miofibra-Movimento nuclear para a periferia-Rigidez nuclear-Reticulação de miofibrilas-Lamina A/C-Desmina

Abstract

Nuclear movements are important for multiple cellular functions and are driven by polarized forces generated by motor proteins and cytoskeleton. During skeletal muscle development and regeneration, nuclei move from the center to the periphery of the myofiber. Moreover, nuclear movement to the periphery begins with the emergence of a nuclear wrinkle between myofibrils that gradually increases forming a bud. The nucleus undergoes severe deformations while being squeezed by myofibrils until it is finally expelled to the periphery. Nuclear positioning at the periphery of myofibers is crucial for proper muscle function, with centrally located nuclei being linked to several muscle disorders. Here we demonstrate that nuclear movement to the periphery of myofibers is dependent on local changes in nuclear stiffness regulated by Lamin A/C. Furthermore, we found that this movement is mediated by Desmin dependent myofibril crosslinking and zipping, while Plectin, Arpc5L and γ actin are necessary for proper Desmin organization at the z-lines. Finally, Nesprin1 depletion resulted in a severe decrease of peripheral nuclei which suggests that it might play a role in nuclear movement to the periphery possibly associated with mechanotransduction.

Keywords: Myofiber-Nuclear movement to the periphery-Nuclear stiffness-Myofibril crosslinking-Lamin A/C-Desmin

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List of abbreviations, acronyms and symbols

BAF – Barrier to autointegration factor

CH – Calponin homology

CNM – Centronuclear myopathies

EC coupling – Excitation-contraction coupling

FRAP – Fluorescence recuperation after photobleaching

FRET – Forester resonance energy transfer

IF – Intermediate filament

KASH – Klarsicht, ANC-1 and Syne homology

LBR – Lamin B receptor

LEM – LAP2-Emerin-MAN1

LINC – Linker of nucleoskeleton and cytoskeleton

MuSK – Muscle specific receptor tyrosine kinase

Nesprins – Nuclear envelope spectrin repeat protein

NLS – Nuclear localization signal

PFA – Paraformaldehyde

PH – Pleckstrin homology

PI3P – Phosphatidylinositol 3-phosphate

PR – Proline rich

SUN – Sad1 and UNC-84

TTN – Titin encoding gene

1. Introduction

1.1. Skeletal muscle cell

Skeletal muscle cells, also called skeletal muscle fibers, are long, cylindrical and multinucleated cells and the main components of muscle. They usually present a diameter between 10 and 100 μm and a length that can extend up to 30 cm. These cells possess a large quantity of myoglobin which can store oxygen similarly to hemoglobin and glycogen granules called glycosomes that provide glucose to the cell, both of which vital for muscle cell activity. Some structures such as myofibrils, sarcoplasmic reticulum and T-tubules are specific to this type of cells and play an important role in muscle contraction (Elaine N. Marieb and Katja N. Hoehn, 2015).

When observing a muscle fiber it is possible to see striations, a sequence of lighter and darker bands called I bands and A bands respectively. The I band has a darker area called the Z disc or Z line while the A band has a lighter area called H zone which in turn is divided by a darker M line, shown in Figure 1.1.

Skeletal muscle fibers are composed of rodlike structures called myofibrils longitudinally distributed throughout each fiber. The number of myofibrils per muscle fiber varies with its size, though they are always densely packed. Desmin, an intermediate filament (IF), has a role of crosslinking myofibrils. Myofibrils contain the contractile units of the muscle, the sarcomeres. A sarcomere is the region of the myofibril comprised between two consecutive Z lines and it is composed of two different myofilaments. Thick filaments containing myosin that are restricted to the A band whereas thin filaments containing actin extend along all the I band and into a small area of the A band (Figure 1.1.) with the latter ones being anchored at Z-lines by α-actinin. In the areas where the myofilaments overlap each thick myosin filament is surrounded by 6 thin actin filaments and each of these is flanked by three thick filaments (Elaine N. Marieb and Katja N. Hoehn, 2015).

The myosin molecule is constituted by two heavy polypeptide chains which compose the rodlike tail and four light polypeptide chains that compose the two globular heads (Fig. 1.1.). The rodlike tails form the central part of the filament while the globular heads, responsible for linking thick and thin filaments forming cross bridges during muscle contraction, are facing outward at the end of the filaments. Actin monomers called globular actin polymerize to form the actin filaments that, when intertwined, compose thin actin filaments (Fig. 1.1.)(Elaine N. Marieb and Katja N. Hoehn, 2015).

Even though this interaction between thick and thin filaments is the engine for muscle contraction, such process would be impossible without the regulatory role played by troponin and tropomyosin. Troponin is composed by three globular polypeptides with three distinct roles: binding actin and

therefore impeding the linking with myosin; binding calcium ions after calcium release due to an action potential which cause troponin to detach from actin allowing the formation of the cross bridges; and binding tropomyosin aiding its binding to actin. Tropomyosin is rod shaped protein that helps stiffen and stabilize actin but when the muscle is relaxed it binds and blocks myosin binding sites stopping the formation of cross bridges (Elaine N. Marieb and Katja N. Hoehn, 2015).

Titin is the protein that forms the core of the thick filaments, anchoring them to the Z lines and M lines maintaining A band organization and helping muscle resist excessive stretching and recover after contraction (Fig. 1.1.). Thin filaments are anchored to integrin proteins in the sarcolemma (plasma membrane) through dystrophin.

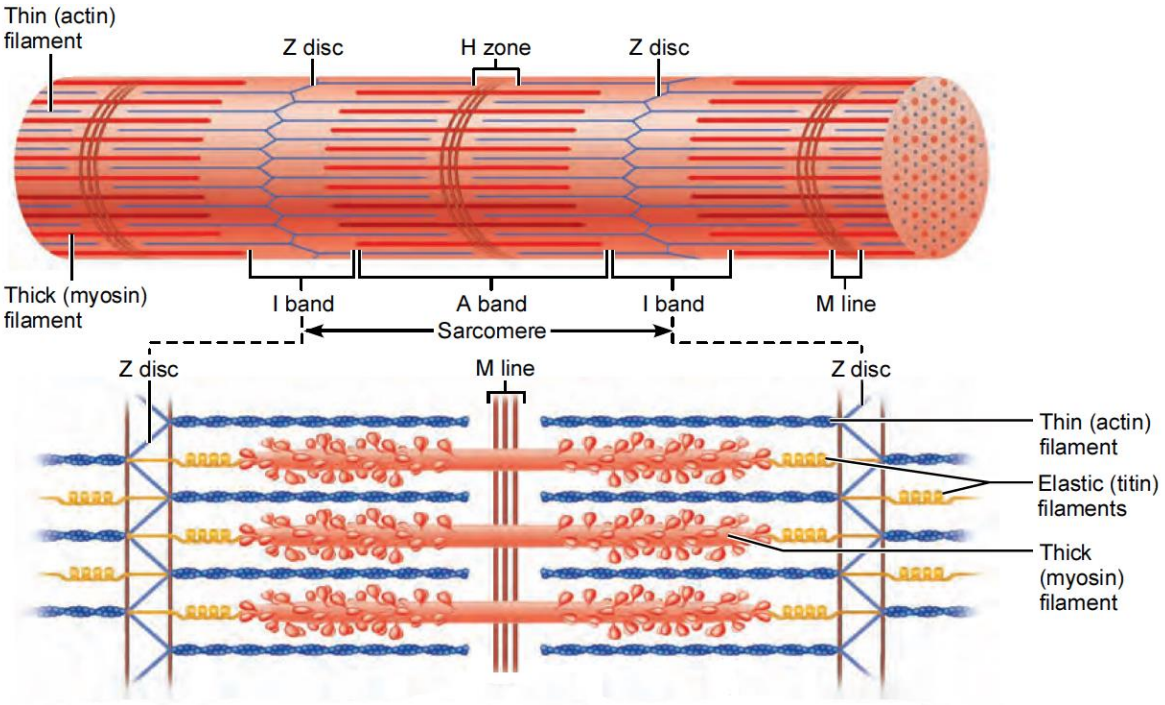


Figure 1.1. Schematic representation of a sarcomere and its structure. Adapted from (Elaine N. Marieb and Katja N. Hoehn, 2015)

According to the sliding filament model of contraction, thin actin filaments slide past thick myosin filaments and overlap to a greater extent during muscle contraction. When stimulated by the motor nerve, thick filaments link to myosin binding site of thin filaments forming the cross bridges and beginning simultaneous contraction of all sarcomeres. During this, cross bridges are formed and destroyed several times increasing the overlap of the filaments and increasing tension bringing thin filaments closer to center of the sarcomere. When contraction ends, cross bridges become inactive and there is a decrease in tension making the sarcomere return to its original relaxed state (Elaine N. Marieb and Katja N. Hoehn, 2015).

The sarcoplasmic reticulum and T-tubules also play a pivotal role in skeletal muscle cell contraction regulation (Fig. 1.2.). The first is very similar to smooth endoplasmic reticulum with a tubule network adjacent to each myofibril which is responsible for the regulation of intracellular ionic calcium levels. This network also has terminal cisternae that flank T-tubules at the A band-I band junction (Fig. 1.2.). Calcium is released when an action potential stimulates a muscle fiber to contract (Elaine N. Marieb and Katja N. Hoehn, 2015).

The plasma membrane of the myofiber (sarcolemma) forms invaginations at the A band-I band junction and forms the T-tubules which increase the muscle fiber surface area and since they are contiguous with the sarcolemma it facilitates the propagation of the impulse (Fig. 1.2.). T-tubules stretch in between two terminal cisternae and form structures called triads, where T-tubule proteins function as voltage sensors and regulate calcium release from the terminal cisternae of the SR to the whole fiber (Fig. 1.2.). These structures are vital for simultaneous calcium release along the fiber ensuring proper signal transmission from the sarcolemma to reach the myofilaments and therefore precise contraction progression. This whole mechanism is called excitation-contraction coupling (EC coupling) (Elaine N. Marieb and Katja N. Hoehn, 2015).

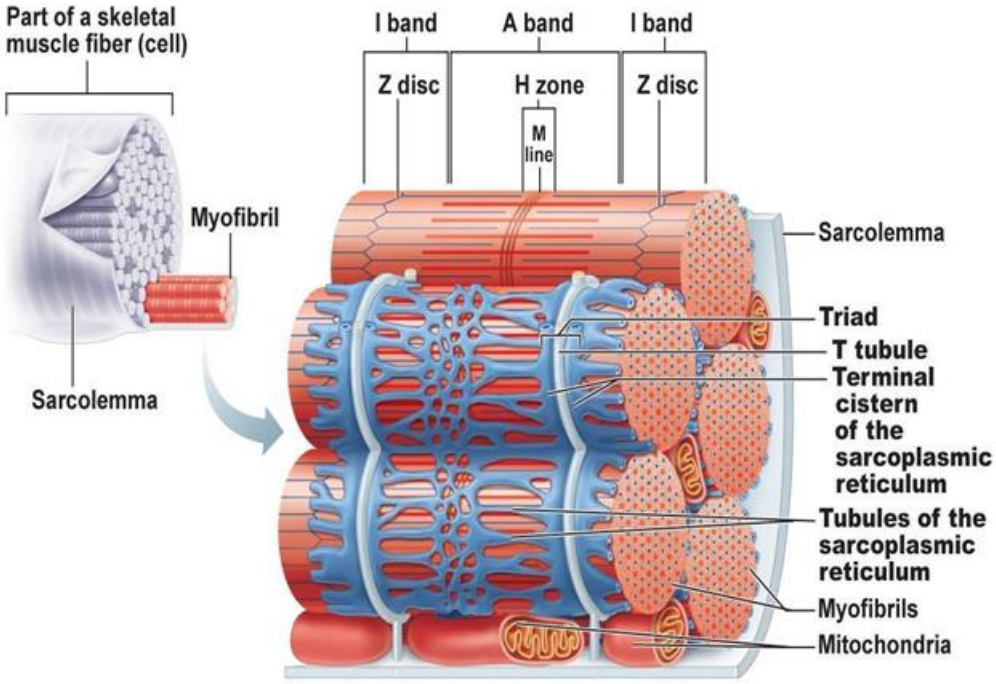


Figure 1.2. Schematic representation of the membrane structures surrounding myofibrils. Adapted from Elaine N. Marieb and Katja N. Hoehn, 2015.

Skeletal muscle cell differentiation

Each muscle fiber results from the fusion of hundreds of myoblasts which need to exit cell cycle in order to gain the ability to fuse with each other (Abmayr and Pavlath, 2012). Initially they form small myotubes that lack the major structures characteristic of a developed myofiber, such as triads (Fig. 1.3.). Upon fusion with a myotube, the myoblast nucleus moves towards the center of the myotube, driven by microtubules (Cadot et al., 2012). This nuclear centration movement is regulated by the small Rho GTPase Cdc42 and Par3 and Par6 polarity proteins with dynein/dynactin motor complex also playing an important role. It is predicted that this movement results from nuclei pulling the microtubules anchored in other nuclei through action of dynein/dynactin (Cadot et al., 2012; Wilson and Holzbaur, 2012).

During myotube development, nuclei spread evenly throughout the longer axis of the myotube in a microtubule dependent movement. In this case, nuclei move considerably slower and it is possible to observe pausing events and nuclear rotation during this movement (Cadot et al., 2012; Englander and Rubin, 1987; Roman et al., 2016 under revision). Three different mechanisms have been proposed to be responsible for nuclear spreading, the first one relying on kif5b/kinesin-1 interaction with microtubule associated protein MAP7. Microtubules anchored at the nuclear envelope by their minus ends form an antiparallel network which is maintained by kinesin-1/MAP7 complex. The force exerted by kinesin-1 moving towards the plus end of microtubules pushes nuclei apart (Bruusgaard et al., 2003; Metzger et al., 2012). Kinesin-1 was reported to localize to the nuclear envelope through its binding to Nesprin2 bound KLC-2 and therefore it was hypothesized that this would be responsible for nuclear rotation during nuclear spreading (Wilson and Holzbaur, 2012; Wilson and Holzbaur, 2015). Another mechanism involves the motor protein dynein anchored at microtubule poles and capable of pulling the microtubules. Dynein also presents a similar function to kinesin-1 when anchored to the nuclear envelope, influencing nuclear rotation during nuclear movement (Folker et al., 2012).

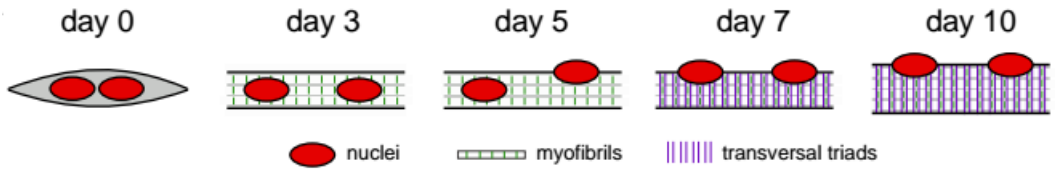


Figure 1.3. Timeline of muscle differentiation in the in vitro system used to study peripheral nuclear positioning and transversal triad formation. Nuclei are in red, myofibrils in white (with z-lines in green) and transversal triads as purple lines. Day 3: myofibril formation. Day 5: initiation of peripheral nuclear positioning. Day 7: transversal triad formation. (Adapted from Roman et al., 2016 under revision).

After nuclear spreading, these centrally located nuclei begin their movement towards the periphery of the muscle fiber (Fig. 1.3., 1.4.) (Shichiji et al., 2013; White et al., 2010; Harris et al., 1989). This process is not exclusive to muscle development since it also happens after injury as a part of a repair mechanism that involves nuclear movement to the center of the fiber and back to the periphery (Pastoret and Sebille, 1995; Maxwell et al., 1984). It was recently shown by our laboratory that nuclear movement to the periphery of skeletal muscle cells is an actin and Nesprin dependent process (Falcone et al., 2014). N-Wasp is an actin nucleation factor pivotal for nuclear movement to the periphery and functions downstream of amphiphysin-2, a protein involved in T-tubule and triad formation (Falcone et al., 2014). The role actin plays in this mechanism suggests the involvement of linker of nucleoskeleton and cytoskeleton (LINC) complex proteins, Nesprin and Sad1 and UNC-84 (SUN) proteins, in the movement to the periphery, in addition to anchoring nuclei at the periphery of the myofiber (Lei et al., 2009; Elhanany-Tamir et al., 2012; Zhang et al., 2010). Desmin was also reported to play a part in nuclear positioning, responsible for maintaining the distance between nuclei (Ralston et al., 2006; Chapman et al., 2014).

The movement of the nucleus to the periphery of the cell begins with the emergence of an elongated nuclear wrinkle through a narrow gap in between myofibrils (Fig. 1.4.). This wrinkle gradually increases in size forming a bud, with the nucleus undergoing dramatic deformation until it is finally expelled to the periphery of the cell (Fig. 1.4.). Before this movement takes place, there is an area near the nucleus devoid of myofibrils. As nuclear movement to the periphery ensues, this area starts to decrease in size as though myofibrils zip together towards the nucleus (Roman et al., 2016 under revision).

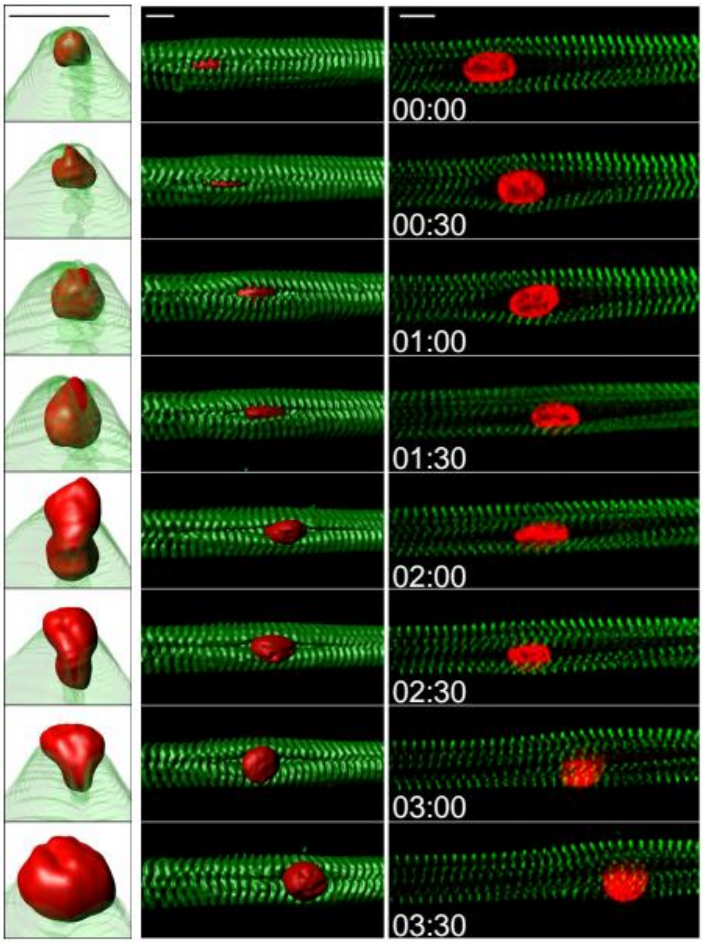


Figure 1.4. Kymograph from a time-lapse movie of a 5-day myofiber depicting peripheral movement of a nucleus (H2B-iRFP, red) through myofibrils (YFP-α-actinin, green). Left: view from the right side, with transparent myofibrils three-dimensional rendering. Middle: view from the top, surface three-dimensional rendering. Time, hh:mm. Scale bar, 10 μm. Right: 2D view of the central plane of a kymograph from a time-lapse movie of a 5-day myofiber depicting peripheral movement of a nucleus (H2B-iRFP, red) through myofibrils (YFP-α-actinin, green). Scale bar, 10 μm.(Roman et al., 2016 under revision)

After nuclear movement to the periphery, the myofiber enters the final stages of differentiation at which time transversal triads are formed and nuclei start to cluster in the neuromuscular junction (Fig. 1.3.)(Merlie and Sanes, 1985). These neuromuscular junctions are mainly formed in the central region of muscles where axons specifically connect due to the clustering of acetylcholine receptors in that area. This process depends on the complex formed between muscle specific receptor tyrosine kinase (MuSK) and LRP4 regulated by agrin; the myoclustering mechanism itself might rely on MuSK patterns in association with Nesprins at neuromuscular junctions (Kim and Burden, 2008; Apel et al., 2000).

1.2. Lamins

IFs protein family is composed by 73 members distributed through 5 distinct groups based on assembly properties, structure and their expression pattern throughout the different tissues (Fig. 1.5) (Eriksson et al., 2009). Type I and II IFs are keratins and they always form heteropolymers while type III IFs, like Desmin, form homopolymers. Neurofilaments like nestin belong to type IV IFs group while nuclear Lamins are type V IFs and type VI IFs group is composed by eye lens proteins, such as Phakinin and filensin (Coulombe and Wong, 2004).

Lamins are the only nuclear IF proteins, responsible for providing structural stiffness and transcription regulation at the nuclear envelope (Stuurman et al., 1998). These proteins also influence nuclear pore positioning as well as nuclear envelope protein anchoring and positioning (Zuleger et al., 2011). Mammals have three Lamin genes which are *LMNA*, *LMNB1* and *LMNB2*. *LMNA* gene encodes all type A Lamins, both major splicing variants Lamin A and Lamin C and the minor splicing variants AΔ10 and C2. Type B Lamins are encoded by two genes; *LMNB1* which encodes one major isoform Lamin B1 and *LMNB2* which encodes the other major isoform Lamin B2 and the minor isoform Lamin B3. B-type Lamins are ubiquitously expressed while A-type Lamins are predominantly expressed in differentiated cells (Butin-Israeli et al., 2012).

Both A and B-type Lamins are composed of an N-terminal globular head, a central alpha helical rod domain and C-terminal tail domains. The latter contains a nuclear localization signal (NLS) and an Ig fold domain possibly involved in protein-protein interactions (Dhe-Paganon et al., 2002; Shumaker et al., 2008). *In vitro*, the alpha helical domains of Lamin monomers interact with each other to form head-to-head dimers, which in turn form a head-to-tail polymer structure. These polymers then assemble side by side in an anti-parallel fashion to create a 5 to 6 μm protofilament, which is the main assembly unit of the Lamin meshwork (Fig. 1.5.) (Foeger et al., 2006; Ben-Harush et al., 2009). Despite this, little is known about the assembly process *in vivo*. Given that Lamins go through extensive posttranslational modifications and interact with the nuclear membrane, chromatin and a vast number of proteins, there are a lot of factors that were not taken into account during the in vitro studies and perhaps play a crucial role in Lamin assembly. *In vivo*, A- and B-type Lamins form separate filament networks with different function, unlike in vitro where they can coassemble. Regardless of their differences in mechanic and biochemical functions, these filament networks are not independent, having several contact points and interacting with each other (Shimi et al., 2008). In terms of mechanical properties, A-type Lamins provide nuclear stiffness enabling nuclei to resist mechanical stress while B-type Lamins are responsible for providing elastic properties which allows nuclei to deform to a certain extent (Broers et al., 2004; Lammerding et al., 2006). Changes in the levels of either A- or B-type of Lamins will result in different nuclear properties. For instance, high Lamin A to Lamin B ratios are associated with increased nuclear stiffness, hindering nuclear

movement and cell migration while low levels of Lamin A make nuclei more susceptible to ruptures (De Vos et al., 2011; Vargas et al., 2012). Furthermore, recent studies suggest that Lamin A levels might be regulated by force transmission via LINC complex and affect the differentiation process (Swift et al., 2013).

Lamins interact with a large number of proteins in the nuclear envelope and the nucleoplasm. In addition to LINC complex proteins, Lamin B receptor (LBR) and LAP2-Emerin-MAN1 (LEM) domain proteins are also known to interact with Lamins (Fig. 1.5.)(Schirmer et al., 2003). LBR is an inner nuclear membrane transmembrane protein that binds heterochromatin and is able to impacts gene silencing mechanisms. In mammals there are 5 LEM proteins in the nucleus: Emerin, LAP2 which has two main isoforms LAP2α and LAP2β, MAN1, LEM2 and LEMD1; most of them requiring Lamin A for their proper localization (Brachner and Foisner, 2011). These proteins also possess the ability to bind to heterochromatin, which, depending on the LEM domain protein, might happen directly or indirectly. LAP2 is able to tether DNA directly due to a LEM like motif while MAN1 and LEM2 are able to do it due to a winged helix motif in the C-terminal domain (Caputo et al., 2006). Indirect binding of DNA is achieved by all LEM domain proteins through interactions between a chromatin binding protein called barrier to autointegration factor (BAF) and the LEM domain (Cai et al., 2001). One of the LAP2 isoforms, LAP2α, lacks the transmembrane domain and localizes to the nucleoplasm where it interacts with both chromatin and A-type Lamins (Brachner and Foisner, 2011). Moreover this particular LEM domain protein might affect cell cycle and chromatin organization (Dorner et al., 2006). Even though some molecular mechanisms behind chromatin tethering to Lamins are known, there is still little data indicating that these mechanisms influence gene silencing.

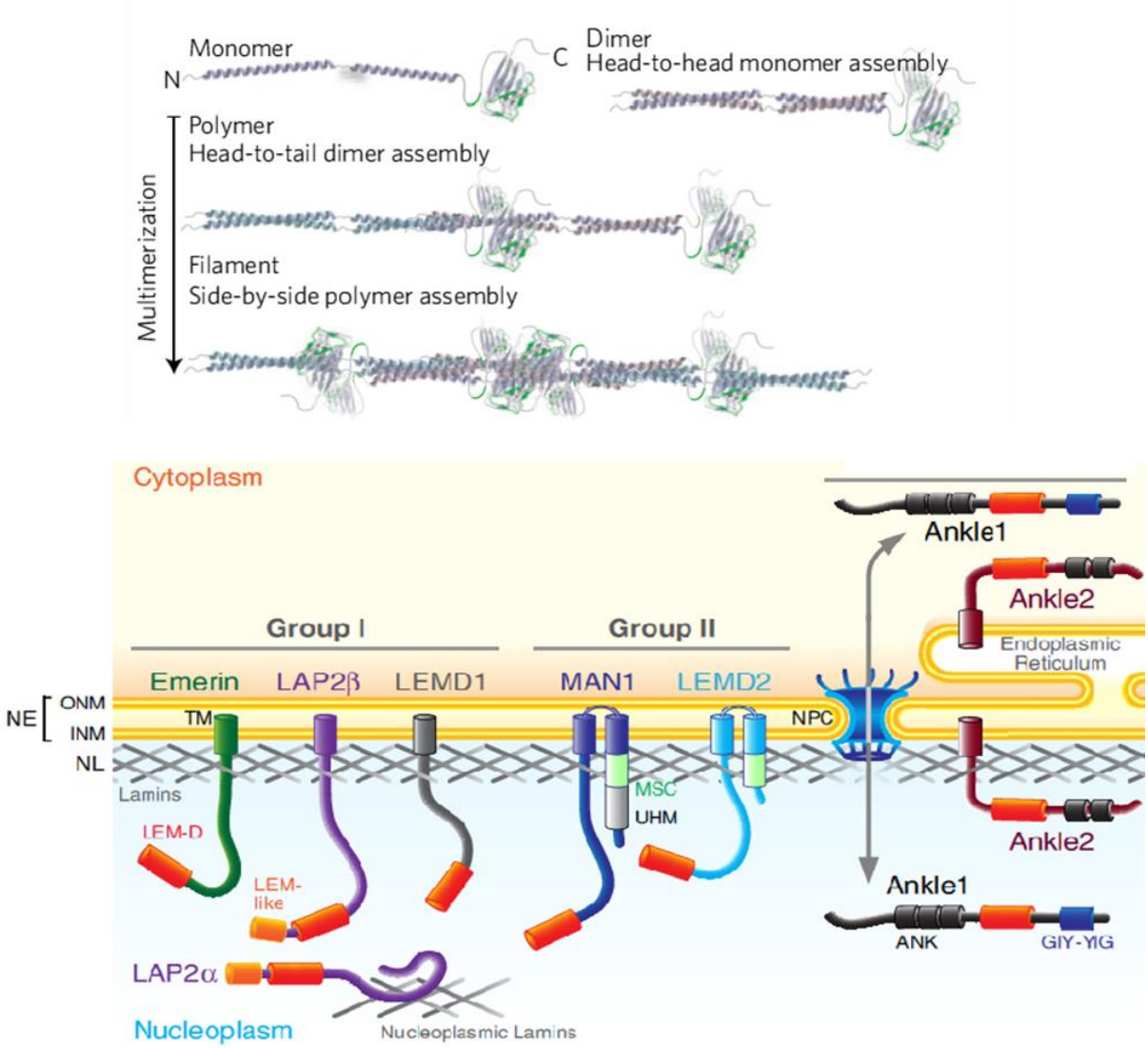


Figure 1.5. Top: Schematic representation of the Lamin protein structure and the assembly process. Adapted from Ihalainen et al., 2015. Bottom: Schematic representation of the nuclear envelope showing Lamin meshwork and LEM domain Lamin-binding proteins. Adapted from Barton et al., 2015.

1.3. LINC complex

The LINC complex is composed of outer nuclear membrane Klarsicht, ANC-1 and Syne homology (KASH) domain proteins and inner nuclear membrane SUN domain proteins (Fig. 1.6.). This complex provides a mechanical link between the nucleus and the cytoskeleton and plays a role in nuclear movement and positioning, mechanotransduction and chromosomic movement (Burke and Roux, 2009; Starr and Fridolfsson, 2010). KASH domain proteins, also known as Nesprins (Nuclear envelope spectrin repeat protein), extend from the cytoplasm, where they bind to cytoskeleton

elements, to the perinuclear space where the KASH domain interacts with SUN proteins (Fig. 1.6.) (Sosa et al., 2012). SUN proteins on the other hand are located in the inner nuclear membrane where they anchor the LINC complex to the nuclear lamina through interaction with A type Lamins and other proteins like Emerin, while the SUN domain interacts with Nesprin KASH domain in the perinuclear space (Fig. 1.6.) (Sosa et al., 2012). SUN2 in particular, multimerises to forms a trimer with a triple helical coiled coil and a globular head, essential for KASH domain to bind along a hydrophobic groove in between SUN domains (Sosa et al., 2012; Zhou et al., 2012). The existence of numerous interactions between KASH and SUN proteins demonstrates how the LINC complex is capable of resisting the mechanical forces applied on the nucleus. There are several Nesprin isoforms that differ in length and their functional domains, with specific Nesprin isoforms bind to specific cytoskeletal elements like actin, microtubules and even IFs (Starr and Fridolfsson, 2010). The giant isoforms Nesprin 1G and Nesprin 2G bind directly to actin filaments through their calponin homology (CH) domains and are necessary for nuclear movement and positioning at the periphery (Starr and Han, 2002). Nesprins that interact with microtubules usually do so through kinesin or dynein motor proteins (Starr and Fridolfsson, 2010). Nesprin1 and Nesprin2 directly bind to dynein through specific regions of their cytoplasmic domains while Nesprin4 binds directly to kinesin light chains and plays an important role in the early development of myofibers, more specifically in nuclear centration and spreading (Roux et al., 2009). The only Nesprin isoform known to bind IFs is Nesprin 3α, that binds Plectin's actin binding domain which in turn binds IFs through the plakin domain (Wilhelmsen et al., 2005). Nesprin3α is also known to bind Nesprin1G actin binding domain possibly exerting some sort of control in nuclear size through the formation of a Nesprin meshwork (Wilhelmsen et al., 2005).

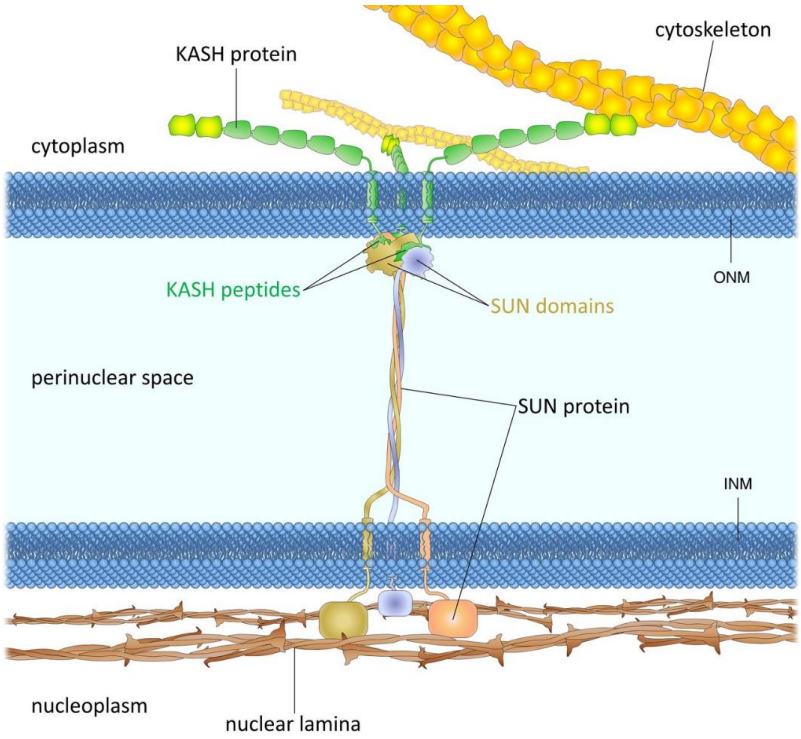


Figure 1.6. LINC complex structural organization and binding partners. KASH proteins bind to cytoskeletal elements such as microtubules actin filaments and IFs while SUN proteins anchor the complex at the INM and interact with the Lamin meshwork. Adapted from Chang et al., 2015.

1.4. Desmin

Mature myofibers possess an IF cytoskeleton which is mainly composed by Desmin. The IFs are mainly localized at the Z lines or associated with the sarcolemma in structures called the costameres (Fig. 1.7.) (Lazarides and Hubbard, 1976). After myoblast fusion and during myotube elongation, desmin interacts with vimentin to form longitudinal strands along the myotube, which after development give rise to transversal filaments localized to the Z lines (Barbet et al., 1991). It was reported that desmin depletion affects myoblast fusion hindering myotube formation, however results obtained in mouse myofibers lacking the Desmin gene suggest that Desmin is not essential for myotube differentiation since they still develop normal myofibers (Schultheiss et al., 1991). This suggests that there might be another IF that can compensate the lack of Desmin.

In mature skeletal muscle fibers, Desmin forms scaffolds around the myofibrils at the Z lines and connects myofibrils to the sarcolemma at the costameres, where Desmin is linked to Plectin. γ-actin links the costameres to the contractile units due to its capability of binding dystrophin at the level of the sarcolemma (Rybakova et al., 2000). Desmin filaments also link several organelles like the nucleus or mitochondria to the sarcomeres at the Z lines. This is achieved through different Plectin isoforms

that make the crosslink between Desmin and organelles, possibly influencing organelle positioning in the fiber as well as nuclear shape and positioning (Konieczny et al., 2008). Nevertheless, it is not certain that nuclear movement to the periphery depends on Desmin transversal scaffold organization.

1.5. Plectin

Plectin is a cytolinker protein, responsible for the anchoring IFs to several cellular structures such as Z lines, costameres and nuclei (Fig. 1.7.). This protein binds to several different IFs from all subgroups, being Desmin and Lamin B interactions noteworthy for nuclear positioning in muscle fibers (Fig. 1.7.) (Foisner et al., 1991; Reipert et al., 1999) . It was previously shown that these interactions are mainly regulated by phosphorylation, with Plectin phosphorylation triggering its dissociation from the binding partner. Phosphorylation of the binding partner might also promote this dissociation as is the case with Lamin B and Plectin interaction (Herrmann and Wiche, 1987; Foisner et al., 1991). Even though its main function is to bind IFs providing structural stability to this particular network, Plectin is also know to bind microtubules and actin (Herrmann and Wiche, 1987; Foisner et al., 1995). This might also help stabilize these cytoskeletal structures and perhaps influence their dynamic regulation, since Plectin deficiency conditions microtubule dynamics and actin filament polymerization. Plectin is widely expressed, however it presents higher levels of expression in cells under great mechanical stress, like muscle cells (Wiche et al., 1983). The Plectin gene contains 41 exons encoding an actin binding domain and a plakin domain in the N-terminal region, a central coiled coil domain and the C-terminal domain. The small N-terminal domain is a variable region between isoforms and defines their subcellular localization (Wiche et al., 1991). One of these isoforms, Plectin1, is localized to the nuclear envelope/endoplasmic reticulum where it is predicted to bind Nesprin3α (Ketema et al., 2007). A study developed in primary dermal fibroblasts from Plectin1 deficient mice showed actin cytoskeleton abnormalities and impaired migration, which in turn suggests an involvement of this isoform in nuclear positioning mechanisms (Abrahamsberg et al., 2005). Alongside Plectin1, Plectin1d isoform, which localizes to the Z lines (Fig. 1.7.), might also play an important role in nuclear positioning in muscle cells.

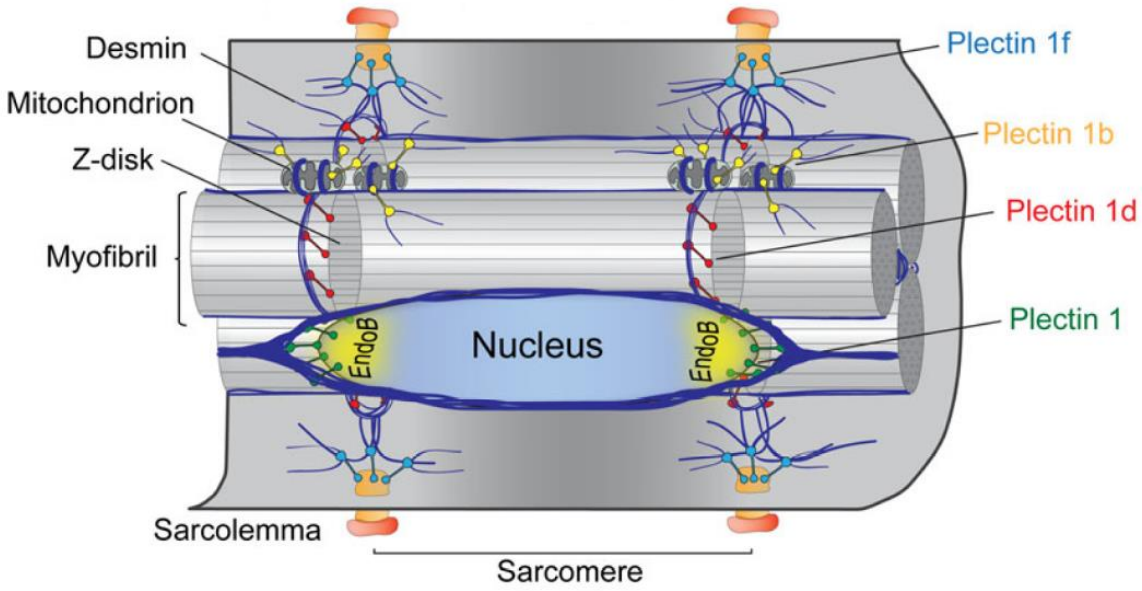


Figure 1.7. Desmin and Plectin subcellular localization and predicted organization in a myofiber. Adapted from Staszewska et al., 2015.

1.6. Centronuclear Myopathies

Centronuclear myopathies (CNM) are a diverse group of neuromuscular disorders that are characterized by centrally positioned nuclei, muscle weakness and atrophy (Fig. 1.8.) (Pierson et al., 2005; Nicot et al., 2007). There are three different genetic forms of this disorder: The X-linked form, that occurs due to mutations in the gene encoding myotubularin, *MTM1* (Laporte et al., 1996); the autosomal-dominant which is caused by mutations in the amphiphysin-2 and dynamin-2 genes *BIN1* and *DNM2* respectively (Bitoun et al., 2005); and the autosomal-recessive caused by mutations in skeletal muscle ryanodine receptor, titin and also amphiphysin-2 encoding genes (*RYR1*, *TTN* and *BIN1* respectively) (Nicot et al., 2007).

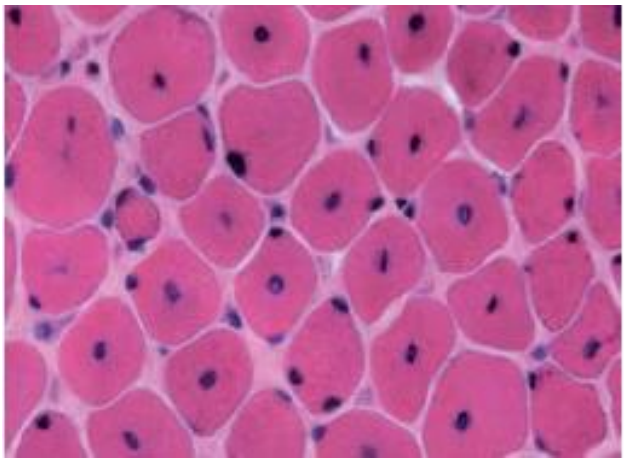


Figure 1.8 Transversal cut of a deltoid muscle. Most nuclei are centrally located which is characteristic of this disorder.

1.6.1. *MTM1* related CNM

The X-linked CNM is the most common form and presents a severe phenotype characterized by neonatal onset, muscle weakness, atrophy and accompanying respiratory involvement with the necessity of invasive respiratory and nasogastric tube feeds in most cases (Herman et al., 1999). This disorder affects 2/100000 male births and is usually fatal within the first year of life, although there are some milder cases in which the individual survives until adolescence or even adulthood.

Myotubularin family of phosphoinositide phosphatases is composed by 14 members in humans, with several of these proteins mutated in neuromuscular diseases or associated to other conditions like metabolic disorders and cancer, with mutations in *MTM1* being the cause for X-Linked CNM (Begley and Dixon, 2005; Lorenzo et al., 2005). To date, more than 300 mutations in *MTM1* have been reported, most of them resulting in a significant reduction of myotubularin protein (Biancalana et al., 2003; Tsai et al., 2005).

These proteins are responsible for the dephosphorylation of phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 3,5-phosphate, both of which are essential second messengers in membrane trafficking (Backer, 2008; Blondeau et al., 2000). The influence of PI3P regulation exerted by myotubularin is not exclusive to membrane trafficking and endocytosis; it also affects autophagy, formation of autophagosomes and autophagosome-lysosome fusion which depend on PI3P synthesis (Cebollero et al., 2012; Funderburk et al., 2010). Abnormalities in muscle autophagy, T-tubule, sarcoplasmic reticulum and triad formation have been reported in several animal models for X-Linked CNM as common downstream consequences of myotubularin deficiency (Dowling et al., 2010).

1.6.2. *BIN1* related CNM

Amphiphysin 2 related CNM is a rare condition restricted to a few families that is caused by recessive mutations. The phenotype is usually mild and it is characterized by progressive muscle weakness and atrophy starting from a young age, although homozygous mutations have been reported to present a lethal phenotype. Alternative splicing of amphiphysin has been associated to myotonic dystrophy, which presents some common features with CNM, particularly centrally located nuclei and triad defects (Fugier et al., 2011).

BIN1 gene encodes amphiphysin 2, a protein localized at the T-tubules and involved in their formation. It has an N-terminal BAR domain involved in membrane binding and an SH3 domain responsible for protein-protein interaction, namely actin nucleation promoting factors (Butler et al., 1997; Lee et al., 2002; Toussaint et al., 2011). N-WASP is an actin nucleation promoting factor known to act downstream of amphiphysin 2 regulating nuclear positioning and triad formation in skeletal muscle fibers. These functions are usually disrupted in cases of CNM since N-WASP is probably misslocalized due to mutations in amphiphysin (Falcone et al., 2014).

1.6.3. *DNM2* related CNM

DNM2 related autosomal-dominant form of CNM presents, in most cases, a milder phenotype than the X-linked and recessive CNM forms and it manifests during adolescence or early adulthood. Identical to other CNM forms, this disorder presents with general muscle weakness which in this case mainly affects proximal muscles. It may also present ptosis with ophthalmoplegia, localized muscle hypertrophy, axonal involvement, neutropenia and cataracts which indicates an influence in other tissues (Liewluck et al., 2010).

Dynamin protein family is composed by three members; dynamin-1 which is mostly expressed in the brain, dynamin-2 which is ubiquitously expressed and dynamin-3 which is expressed in the brain and testes (Praefcke and McMahon, 2004). These proteins are mainly involved in membrane fission and endocytosis but further roles have been proposed in microtubule network, centrosome cohesion and actin cytoskeleton assembly, all of which can explain some of the aberrations in nuclear positioning observed in this condition . Furthermore some abnormalities in autophagy pathways were observed in mouse models of a common human *DNM2* dominant mutation, similar to the ones observed in animal models for the X-Linked CNM form.

Dynamin-2 is a large GTPase with five major functional domains; a C-terminal proline rich (PR) domain, a pleckstrin homology (PH) domain, a GTPase effector domain, a middle domain and an N-terminal GTPase domain (Gu et al., 2010; Thompson et al., 2004). The most severe phenotypes reported about this form have been related to heterozygous *de novo* mutations in the PH domain, which binds to phosphoinositides, while milder phenotypes are associated with middle domain and PR

domain mutations, with the PR domain able to bind to SH3 domain proteins such as amphiphysin (Bitoun et al., 2005; Bitoun et al., 2007). Some studies suggest that *DNM2* mutations hinder N-WASP localization at the triads and therefore triad biogenesis, thus affecting proper muscle contraction (Falcone et al., 2014).

1.6.4. *RYR1* related CNM

This particular form of CNM is caused by recessive mutations in the *RYR1* gene and presents an intermediate phenotype in terms of severity. The phenotype is very similar to other forms referred above, with the particularity of having significantly less respiratory impairment (Wilmshurst et al., 2010). Mutations in this gene are known to be involved in a number of neuromuscular disorders other than CNM and it is not unusual to have more than one pathogenic mutation in the same allele which reflects the complexity of *RYR1* related myopathies (Klein et al., 2012).

Ryanodine receptors are a family of intracellular calcium channels divided in three main isoforms that are tissue specific. Ryanodine receptor 1 is mainly expressed in skeletal muscle cells, ryanodine receptor 2 is primarily expressed in the myocardium while ryanodine receptor 3 is more widely expressed that the first two but has increased expression in the brain (Takeshima et al., 1989; Nakai et al., 1990). These ryanodine receptors mediate calcium release from the sarcoplasmic reticulum and endoplasmic reticulum which is of extreme importance for excitation-contraction coupling in skeletal muscle (Inui et al., 1987).

1.6.5. *TTN* related CNM

This is a very rare form of CNM with only 5 cases of CNM linked to recessive mutations in the titin encoding gene (TTN) so far. Mutations in *TTN* gene are also responsible for other neuromuscular disorders and truncated variants of this protein are relatively common. The 5 individuals with this condition presented generalized muscle weakness, respiratory impairment but with no cardiac involvement (Ceyhan-Birsoy et al., 2013).

Titin anchors thick filaments at the Z and M lines maintaining sarcomere organization (Fürst et al., 1988). In *TTN* related CNM cases, interaction between titin and M lines is usually affected, disturbing sarcoplasmic reticulum linkage through obscurin, which in turn impacts the organization of the sarcomere (Bagnato et al., 2003; Charton et al., 2010). C-terminal truncations are also common in this type of pathology, culminating in a reduction of proteins like nebulin and calpain-3. The latter is vital for the correct localization of ryanodine receptors to the triad structure thus affecting proper EC coupling and therefore contraction (Lange et al., 2009).

1.7. Laminopathies

This group of rare genetic disorders is caused by mutations in Lamin encoding genes. Laminopathies are usually cell type specific but can affect several tissues at the same time with some similarities in the phenotype, for example in progeroid syndromes (Worman and Foisner, 2010). The fact that most laminophaties are tissue specific has a few proposed explanations and yet the mechanism remains elusive. One hypothesis states that mutant Lamins lead to changes in Lamin meshwork structure weakening it and making nuclei more susceptible to mechanical force (Brosig et al., 2010). On the other hand, Lamin mutation might impact overall gene expression during differentiation and it has been shown that Lamin A deficient myoblasts have reduced expression of proteins, such as Desmin, which are essential for muscle differentiation (Columbaro et al., 2005; Furukawa et al., 2009). These disorders most commonly affect striated muscle usually due to mutations in *LMNA* gene (Zaremba-Czogalla et al., 2012), although some Lamin B related laminopathies have been reported (Padmakumar et al., 2005). These mutations can be missense, nonsense, splice site mutations, in-frame and out-of-frame insertions/deletions, however nonsense and small out-of-frame insertions/deletions are very characteristic of muscle related laminopathies. One of the most common muscle related laminopathies is the Emery-Dreifuss muscular dystrophy, which can be caused by mutations in *LMNA* gene as well as mutations in *SYNE1*, *SYNE2*, Emerin (*EMD*) and *FHL1* (Bonne et al., 1999; Gueneau et al., 2009). Nevertheless the same mutation in the *LMNA* gene might present with phenotypic variations which, coupled with the large number of genes involved, suggests the involvement of several major players in this type of laminopathies.

1.8. Desminopathies

Desminopathies are characterized by the existence of Desmin aggregates and deficiencies in sarcomeric organization (Goebel, 1995). Desmin mutations most commonly occur in the alpha-helix intermediate domain and tail domain, with 37 of the 42 reported mutations. Alpha-helix domain mutations usually impair Desmin filament assembly, both in Desmin dimerization and dimer-dimer interaction to form filaments (Kaminska et al., 2004). On the other hand, tail domain mutations have no reported impact in Desmin filament assembly, affecting Desmin interactions with other cytoskeleton components like Plectin instead (Bär et al., 2007; Dalakas et al., 2003). Even so, both types of mutations have very similar and severe phenotypes.

1.9. Plectinopathies

Plectinopathies usually display with muscular dystrophy, skin blistering and neuropathy. The most common disorder associated to Plectin mutations is epidermolysis bulbosa simplex with muscular dystrophy (Gache et al., 1996). Patients suffering from this condition present Desmin aggregates, deficient myofibrils and overall cytoskeletal organization of myofibers similar to what happens in mouse models. Even though there are several Plectin isoforms no correlation between site of mutation and displayed phenotype has been made (Konieczny et al., 2008). Nonsense mutations and out-of-frame insertions/deletions compose the great majority of Plectin mutations, resulting in truncated proteins that downregulate their own mRNA through nonsense mediated mRNA decay (Baker and Condon, 2004). In muscle cells, the phenotype of plectinopathies and desminopathies is very similar since both Plectin and Desmin play major roles in myofiber cytoskeletal organization and might even play a role in nuclear positioning mechanisms.

1.10. Objectives

We recently found that nuclei are moved to the periphery of myofibers by an unexpected mechanism involving the crosslinking and contraction of myofibrils. Furthermore we demonstrated that Arp2/3 complexes containing Arpc5L together with γ-actin are involved in the crosslinking of myofibrils that act as closing zippers on both sides of the nucleus (Roman et al., 2016 under revision). Myofibrils induce growing centripetal forces on centrally located nuclei. These centripetal forces eventually squeeze and extrude the nuclei to the cell periphery (Roman et al., 2016 under revision). We hypothesize that myofibril crosslinkers such as Desmin are regulated by Arp2/3 complexes containing Arpc5L with γ-actin allowing force to be exerted in the nucleus. We also predict that nuclear stiffness, which is mainly regulated by nuclear Lamins, is required for nuclear squeezing to the periphery of the myofiber. In this work we further explore the mechanism of nuclear movement to the periphery of myofibers, focusing on determining the role of myofibril crosslinkers, nuclear stiffness and LINC complex.

2. Materials and Methods

2.1. Myoblast Isolation

All procedures using animals were approved by the Institutional ethics committee and followed the guidelines of the National Research Council Guide for the care and use of laboratory animals. Hind limb muscle *tibialis anterior*, *extensor digitorum longus*, *gastrocnemius* and *quadriceps* from P3-P7 newborn mice were isolated (Figure 2.1) and placed in ice cold Dulbecco's PBS (Sigma-Aldrich® cat# D8537-500ML). Exceeding PBS was removed using a 10 ml pipette in order to facilitate the mincing process. Isolated muscle was minced with a dissection scissor and digested for 1h 30 minutes at 37ºC in 5 ml Digestion mixture (0,5mg/ml collagenase (Sigma-Aldrich® cat: C0130-500MG) and 3.5mg/ml dispase (Invitrogen® cat# 17105041) in Dulbecco's PBS; mixture was then filtered with 0.22 mm Minisart® high flow Syringe Filter(Sartorius cat# 16541-K)). Digestion reaction was stopped by adding 6 ml Dissection medium (IMDM with Glutamax (Invitrogen cat# 31980022); penicillin/streptomycin 1% (Alfagene cat# 15140-122); Fetal Bovine Serum (FBS) 10% (Eurobio cat# CVFSVF00-01) previously heated to 37ºC and the obtained suspension was centrifuged at 600 rpm during 5 minutes. The fat residues and cell debris present in the supernatant were aspirated using a vacuum pump and the suspension was centrifuged again at 600 rpm during 5 minutes. The supernatant was centrifuged at 1400 rpm during 5 minutes after which the supernatant was discarded and the pellet containing the cells was ressuspended in 10 ml of Dissection medium. The obtained cell suspension was filtered using a 40 µm cell strainer (Enzifarma cat# 352340) followed by the addition of 15 ml Dissection medium to reach 25 ml total volume that was then pre-plated on 150 mm petri dishes (SARSTEDT cat# 83.3903) for 3.5 to 4 hours. One hour before the end of pre-plating, fluorodishes (WPI cat# FD35-100) and 35 mm dishes ((LabClinics cat# 153066) number of fluorodishes and 35 mm dishes used in each experiment depends on the number of newborn mice utilized; 1 newborn mouse is equivalent to 2 fluorodishes or 1.5 35 mm dishes) were coated with Matrigel Reduced Factor (Corning cat# 354230) diluted 1:100 in 500 ml of IMDM with Glutamax and left for one hour at room temperature. Matrigel Reduced Factor must be kept on ice during this procedure since it will start to polymerize at 10ºC. After pre-plating, the supernatant of the 100 mm petri dish was collected and centrifuged at 1400 rpm during 5 minutes. The supernatant was then discarded and the pellet was ressuspended in a suitable volume of Growth medium (IMDM with Glutamax; penicillin/streptomycin 1%; FBS 20%; Chicken Embryo Extract (produced in the laboratory); (The suitable volume of Growth medium varies according to the number of mice used in each procedure; suitable volume of Growth medium equal to number of mice used)) keeping the suspension with a higher cell concentration for cell counting, with further addition of growth medium until a concentration of approximately 150000 cells per milliliter is achieved. Subsequently, the Matrigel was discarded, the fluorodishes and 35 mm

dishes were washed with 500 ml Dulbecco's PBS and cells were plated; 150000 in fluorodishes and 225000 in 35 mm dishes.

2.2. Myoblast Differentiation

Primary myoblasts usually take between 2 to 5 days to reach confluence. They start to fuse spontaneously when they reach 70% to 80% confluence and at this time we switched Growth medium with Differentiation medium (IMDM with Glutamax; 2% HyClone Donor Equine Serum (GE Healthcare Life Sciences cat# SH3007402); penicillin/streptomycin 1%). This is done by aspirating the Growth medium and washing once with 1 ml Differentiation medium before adding it. The day after switching mediums we started the full differentiation procedure by putting a Matrigel cryotube containing 1 ml to thaw at 4ºC. After thawing, Matrigel was diluted 1:1 with chilled Differentiation medium and cells were covered with this mixture immediately after medium aspiration (150 µl and 225µl total volume for fluorodishes and 35 mm dishes respectively). The cells were placed in the incubator at 37ºC and 5% CO_2 for 30 to 40 minutes until Matrigel polymerizes. After this, 1 µl of recombinant rat agrin (R&D Systems cat# 550-AG-100) was added per 1 ml of Differentiation medium (previously warmed up to 37ºC) which was then added to the cells (1 ml per fluorodish and 1.5 ml per 3,5 mm dish). Every two days, half the medium was changed by discarding 500 µl and adding 500 µl new Differentiation medium with twice the agrin in order to ensure same agrin concentration. The first day of agrin is considered day 1 of differentiation and fibers take between 7 to 10 days to fully mature.

2.3. Transfections

In muscle cells, the transfection procedure has to be performed at the correct stage of development in order to increase transfection efficacy and also cell survivability. When cells start to fuse and the first, small myotubes start to appear they are ready for transfection. First of all, DNA/RNA and Lipofectamine mixtures were prepared in cryotubes (Fisher Scientific cat# 1000-4220) by adding 1 µl DNA/RNA or Lipofectamine to 49 µl of Opti-MEM medium (Life Technologies cat# 31985-047) respectively, mixed carefully and were then incubated at room temperature for 5 minutes (Each 50 µl of DNA/RNA mixture is prepared in separate cryotubes and used to transfect cells in a single fluorodish; all steps involving pipetting Lipofectamine must be done carefully so that liposome formation is not affected). Following the incubation step, 50 µl of Lipofectamine mixture were added to DNA/RNA mixture and gently mixed; Lipofectamine 3000 (Life Technologies cat# L3000-008)was used for plasmids and co-transfections while Lipofectamine RNAiMAX (Life Technologies cat # 15338-100) was used for siRNA transfection. The mixture containing DNA/RNA and the respective Lipofectamine was incubated at room temperature during 30 minutes. During the waiting period, a

suitable volume of Transfection Medium (IMDM with Glutamax, 1% FBS) was heated up to 37ºC, with 400 µl being required for each cryotube. After the incubation time, 400 µl of Transfection medium are added to each cryotube and Growth medium is switched with the Transfection mixture obtained. Cells were kept in this mixture for 5 hours at which time it was discarded. Cells were washed once with Differentiation medium and 1 ml of Differentiation medium was added immediately after washing. Cells were maintained in culture until full maturation using Myoblast Differentiation protocol as shown before. All siRNAs used are described in Table 2.1.

2.4. Plasmids

YFP-α-actinin plasmid was a gift from Pekka Lappalainen. iRFP-H2B was a gift from Mathieu Coppey. EmGFP-Desmin was obtained through addgene (plasmid #54059). mCherry-Lamin A/C was obtained through addgene (plasmid#55068).

Table 2.1. siRNAs used for protein knockdown.

Target	Ambion ID	Sequence (sense)	Sequence (anti-sense)
Scrambled	genecust	UUCUCCGAACGUGUCACGUtt	ACGUGACACGUUCGGAGAAtt
γ-actin	s61904	AGAUAAUGUUUGAAACCUUtt	AAGGUUUCAAACAUUAUCUgc
β-actin	s200989	UGACGUUGACAUCCGUAAAtt	UUUACGGAUGUCAACGUCAca
Nesprin1	genecust	CCAUCGAGUCUCACAUCAAtt	UUGAUGUGAGACUCGAUGG
Arpc5	s206017	AGAUGAUGCUAUAAGUAtt	UACACUUAUAGCAUCAUCUgg
Arpc5L	s92445	GCGUGGAUAUCGACGAAUUtt	AAUUCGUCGAUAUCCACGCgg
Lamin A/C 1	S69252	GGCUUGUGGAGAUCGAUAAtt	UUAUCGAUCUCCACAAGCCgc
Lamin A/C 2	s69253	CCACCGAAGUUCACCCUAAtt	UUAGGGUGAACUUCGGUGGga
Lamin B1	S69255	GACUUGGAGUUUCGUAAAAtt	UUUUACGAAACUCCAAGUCct
Desmin	s64942	GAGGAGAUCCGACACCUUAAt	UUAGGUGUCGGAUCUCCUCct
Plectin 1	s201801	GGAGUGACCGCAAUACCAAtt	UUGGUAUUGCGGUCACUCCaa
Plectin 2	S201802	CGAGUACACCUUUGAGGGAtt	UCCCUCAAAGGUGUACUCGgg
Plectin 3	s71823	GGCCGUCUCUUCAAUGCUAtt	UAGCAUUGAAGAGACGGCCat

2.5. Immunofluorescence

Immunofluorescence was performed in order to determine protein localization, function in nuclear movement and to do statistical analysis of specific phenotypes. When myofibers reached the desired stage in development they were fixed using 200 µl of 4% paraformadehyde (PFA) (Science Services cat# E15710) for 10 minutes following an initial wash with 200 µl PBS to remove the medium. The

day at which cell were fixed depended on the goal of the experiment. For instance, for quantifications and statistical analysis only cells fixed between day 7 and day 10 of development were used while for protein localization and function in nuclear movement assays cells were fixed at day 4 to 5 of development. After fixing, cells were washed two times with PBS and then permeabilized with a 0.5% triton (Sigma-Aldrich cat# X100-100ML) solution for 5 minutes. The cells were then washed two times with PBS before adding 200 µl of a blocking mixture composed by 50% BSA (5 g BSA (Sigma-Aldrich cat# A7906-50G) in 10 ml MiliQ water) diluted 1:10 in Goat Serum 10% during one hour. Next, cells were washed once with 200 µl of PBS to remove the blocking mixture and the primary antibody solution, composed by 50% BSA and 1% Saponin (0.1 g Saponin (Sigma-Aldrich cat# 47036-50G-F) and 5 g BSA in 10 ml MiliQ water) diluted 1:10 in goat serum 10% plus primary antibodies (diluted 1:5 to 1:200 depending on the primary antibody used), was added. Cells are then left at 4ºC overnight. To remove the primary antibody solution the cells were washed two to three times with PBS for 5 minutes each time. This wash was done with agitation in order to reduce unspecific binding and antibody clustering. Subsequently, 200 µl of the secondary antibody solution was added to the cells and incubated for one hour at room temperature, protected from light with tin foil. This solution was composed by 50% BSA and 1% Saponin diluted 1:10 in goat serum 10% plus secondary antibodies, DAPI and Phalloidin (with secondary antibodies diluted 1:200, DAPI diluted 1:1000 while Phalloidin was diluted 1:200 depending on the phalloidin used). Following this incubation period, the secondary antibody solution was discarded and cells were washed three times with 200 µl of PBS for 10 minutes each. Once again this wash was done with agitation. Finally 200 µl of mounting medium Fluoromout G (Southern Biotech cat# 0100-01) were added to the cells and left overnight to dry before image acquisition. The described immunofluorescence protocol is referent to the staining of only one fluorodish. All antibodies and antibody dilutions are described in Table 2.2 and Table 2.3.

Table 2.2. List of antibodies used in Immunofluorescence and Western Blotting.

Antibody epitope	Species	Company	Concentration
Nesprin 1	Mouse	Courtesy of Dr. Burke laboratory	IF 1:5
α-actinin	Mouse	Sigma (cat #A7732)	IF 1:200
Desmin	Mouse	Dako (cat# Clone D33)	IF: 1:200 WB: 1:1000
Plectin	Rabbit	Sigma (cat# HPA029906)	IF: 1:200 WB: 1:1000
Lamin A/C	Rabbit	Santa Cruz Biotech (cat# sc-20681)	IF: 1:100 WB: 1:1000
Lamin A/C-C	Mouse	Abcam (cat# ab8984)	IF: 1:200
Lamin B1	Rabbit	Abcam (cat# ab16048)	IF: 1:200

Table 2.3. List of secondary antibodies used in Immunofluorescence

Secondary antibodies	Species	Company	Concentration
Anti-Rabbit IgG (H+L) 555 Alexa Fluor	Goat	Life Technologies (cat# A21429)	IF 1:200
Anti-Mouse IgG (H+L) 488 Alexa Fluor	Donkey	Life Technologies (cat# A21202)	IF 1:200
Phalloidin	-	Thermo Scientific (cat# A12379)	IF: 1:200

2.6. Microscopy and Image analysis

Live imaging was performed in a Zeiss Cell Observer fully motorized inverted microscope equipped with a spinning disk confocal unit, a with a large cage incubator and a small stage incubator for temperature control and CO2 supply, a 63x oil immersion objective, a Definite Focus unit and an Evolve 512 EMCCD camera. Cells were maintained at 37ºC and 5% CO_2 in Differentiation medium supplied with agrin during image acquisition which was done at 5 minute intervals for spinning disk confocal microscopy and at 15 minute intervals for widefield. For fluorescence recuperation after photobleaching (FRAP) experiments, cells were photobleached for 5 to 10 minutes by scanning half a nucleus with 100% intensity of 555 nm laser. Acquired images were analyzed using the ZEN software (Blue edition), Fiji and Imaris 8. Confocal images of fixed cells were acquired using a Zeiss LSM 710 and a Zeiss LSM 880 confocal point scanning microscopes both with a 63X oil immersion objective. Images were analyzed using ZEN software (Gray edition), Fiji and Icy. 3D rendering was done with the software Imaris 8, by manually or automatically creating a Surface in the Surpass function. All image acquisition for one specific experiment was done using the same settings for data to be comparable.

3. Results

The theoretical model developed in the laboratory takes into account known biophysical parameters of myofiber components and predicts the role of myofiber contraction, myofibril cross-linking and nuclear stiffness for nuclear movement to the periphery (Fig. 3.1.). In this project, we explore the role of nuclear Lamins, main determinants of nuclear stiffness and plasticity; Desmin, the main myofibril crosslinker; and Nesprins, components of the LINC complex with predicted involvement in this movement (Falcone et al., 2014). Firstly, we tried to determine if Lamin absence and overexpression caused a phenotype through siRNA mediated knockdown and transfection with mCherry-Lamin A/C respectively, followed by fixing and immunofluorescence staining. Furthermore we looked into Lamin distribution throughout the nuclear envelope with further immunofluorescences and FRAP experiments. Desmin and Nesprin function was characterized by siRNA mediated knockdown and overexpression coupled with live imaging and fixed cell imaging with further image analysis.

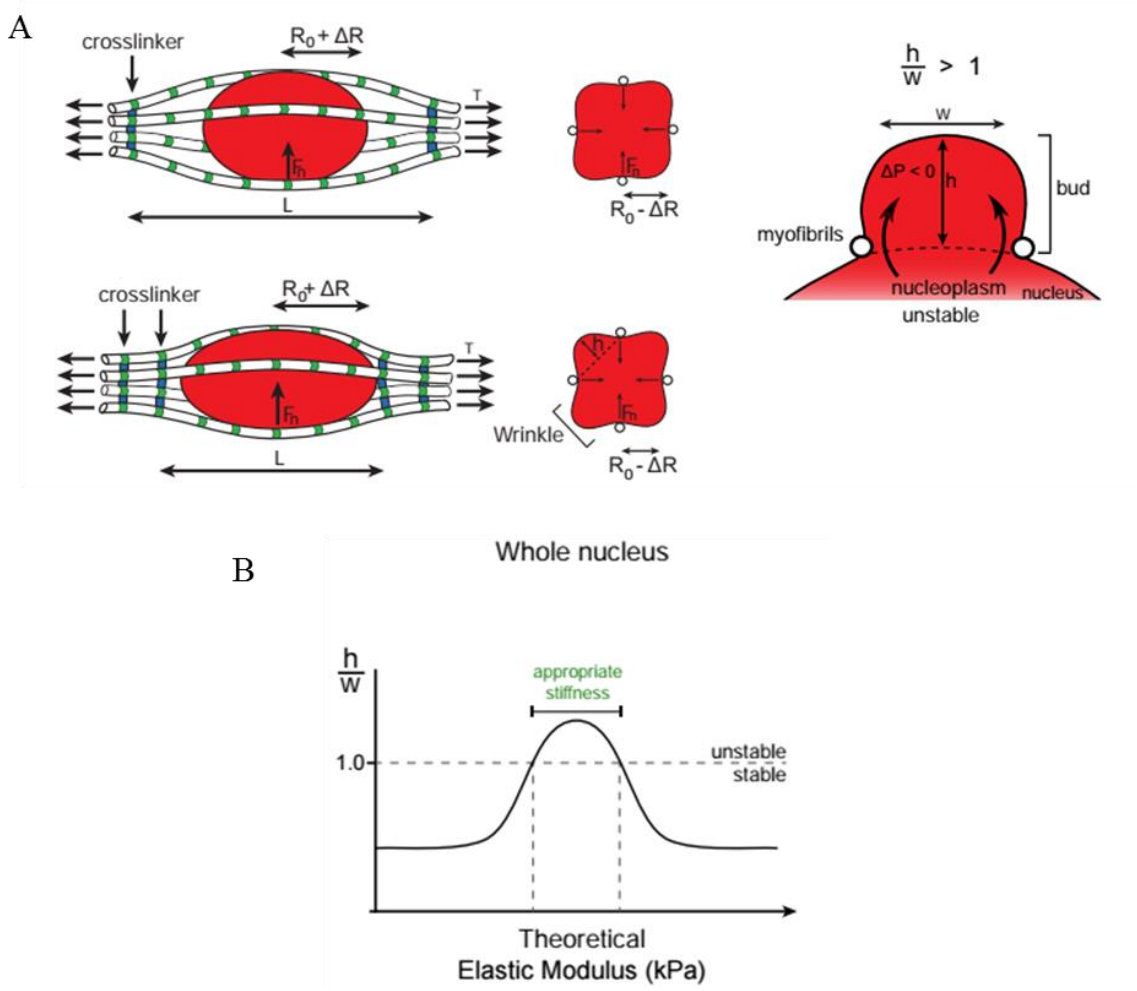


Figure 3.1. Theoretical model of peripheral nuclear movement. A) Schematic of a nucleus during peripheral migration. Longitudinal view (left) and transversal view (right). R0 = radius of the

undeformed nucleus. ΔR = amplitude of radial deformation. Fn = force applied by myofibrils on the nucleus. L = length between the crosslinkers (blue) on each side of the nucleus. h = height of a nuclear wrinkle formed by myofibril pressure. B) Model prediction of the stability of wrinkles relative to global nuclear stiffness. The scaled wrinkle size h/w is plotted as a function of global E.

3.1. Lamins

3.1.1. Nuclear stiffness in nuclear movement to the periphery

The goal was to manipulate nuclear stiffness through Lamin siRNA mediated knockdown and by mCherry-Lamin A/C overexpression to assess possible effects in nuclear movement to the periphery. This was done by transfecting cells at day 0 of differentiation and waiting for them to reach full maturity at day 7 to 10. The cells were then fixed with PFA and stained for Lamin A/C and Phalloidin. We started by transfecting cells with two different LMNA siRNAs in order to test their efficacy and assess the phenotype they originated compared to a negative control. The negative control used was a Silencer Negative control that has no significant similarities with mouse gene sequences, furthermore the influence in cell fusion and development is negligible. One of them (Lamin A/C 2) triggered substantial cell death soon after transfection, which affected myoblast fusion and hindered further cell development. Despite reducing siRNA concentration to half in subsequent experiments, cell death remained a significant issue making it impossible to use in this experiment. On the other hand, Lamin A/C 1 siRNA did not induce such substantial cell death letting myoblasts fuse and differentiate and in turn allowing for proper image acquisition and analysis. One possible explanation for the difference in results between both siRNAs might be the existence of off-target effects in the case of Lamin A/C 2 siRNA that might have led to an increase in cell mortality. For this experiment, only the Lamin A/C 1 siRNA was used. We found that Lamin A/C downregulation leads to a decrease of peripheral nuclei (Fig. 3.2.A, B). The Lamin A/C knockdown was confirmed by immunofluorescence (Fig. 3.2.A) and by western blotting (Fig. 3.2.C). Similarly, Lamin A/C overexpression led to a decrease of peripheral nuclei suggesting that both the increase and decrease of nuclear stiffness impair nuclear movement to the periphery (Fig. 3.2.A, B).

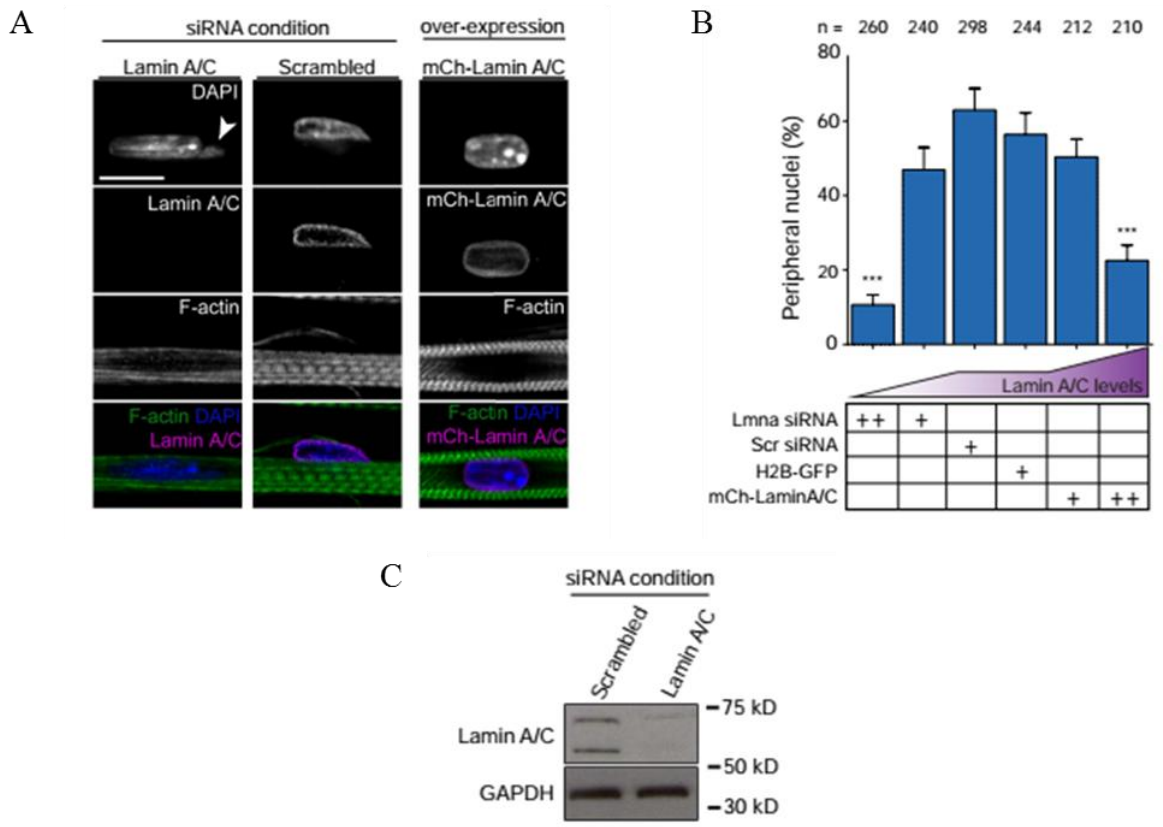


Figure 3.2. Nuclear stiffness is involved in nuclear movement to the periphery. A) Representative immunofluorescence images of 10-day myofibers knocked down for Lamin A/C, scrambled or over-expressing mCherry-Lamin A/C (mCh-Lamin A/C) and stained for Lamin A/C (magenta), F-actin (green) and DAPI (nucleus, blue). Scale bar, 10 μm. B) Quantification of peripheral nuclei positioning in 10-day myofibers knocked down for Lamin A/C or scrambled, or over-expressing H2B-mCherry or mCherry-Lamin A/C. C) Western blot with indicated antibodies from 10-day myofibers knocked down for scrambled or Lamin A/C.

Moreover, Lamin B's function in this mechanism was also studied by siRNA mediated knockdown. For this assay only one Lamin B siRNA was used and its knockdown was confirmed by immunofluorescence alone (Fig. 3.3.A). Although Lamin B expression was downregulated the number of peripheral nuclei did not significantly decrease, which is indicative that Lamin B is not required for proper nuclear migration to the periphery /Fig. 3.3.A, B). Given that Lamin A/C is the main determinant of nuclear stiffness while Lamin B is mostly responsible for nuclear elastoplasticity these results fall within the hypothesis presented by the theoretical model.

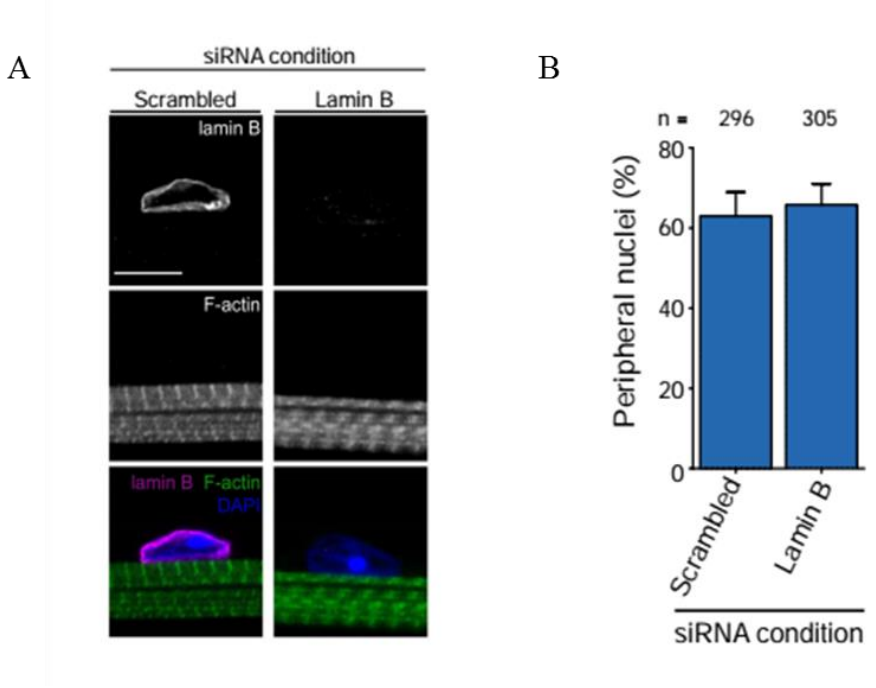


Figure 3.3. Lamin B is not involved in nuclear movement to the periphery. A) Representative immunofluorescence images of 10-day myofibers knocked down for scrambled or Lamin B and stained for Lamin B (magenta), F-actin (phalloidin, green) and DAPI (nucleus, blue). Scale bar, 10 μm. B) Quantification of peripheral nuclei in 10-day myofibers knocked down for scrambled or Lamin B.

3.1.2. Lamin distribution throughout the nuclear envelope during nuclear movement to the periphery

Local alterations of nuclear Lamins could be induced during nuclear movement to the periphery in order to locally alter nuclear stiffness and therefore facilitate this mechanism. This might be of special importance during bud formation. To test if these alterations were occurring, we looked at endogenous levels of Lamin A/C and B. For that we fixed wild type cells at day 5, when nuclei start their movement to the periphery with formation of the bud, and performed immunofluorescence staining for Lamin A/C and B. Using anti-Lamin A/C and anti-Lamin B primary antibodies, the experiments showed that only Lamin A/C was asymmetrically distributed during bud formation (Fig. 3.4.A, B). Furthermore, a reduction of Lamin A/C multimerization was identified in the bud when using an epitope specific Lamin A/C antibody (A/C-C) (Fig. 3.4.A Right panel)(Ihalainen et al., 2015). The epitope of this particular antibody is composed by two separate regions that get into close proximity of each other when they bind to Lamin A/C monomer or dimer, with Lamin multimerization obstructing this binding. Also noteworthy is the fact that this antibody binds to specific Lamin A/C region known to be the binding site of many interacting proteins such as Emerin, SUN1/2, LAP2α and actin.

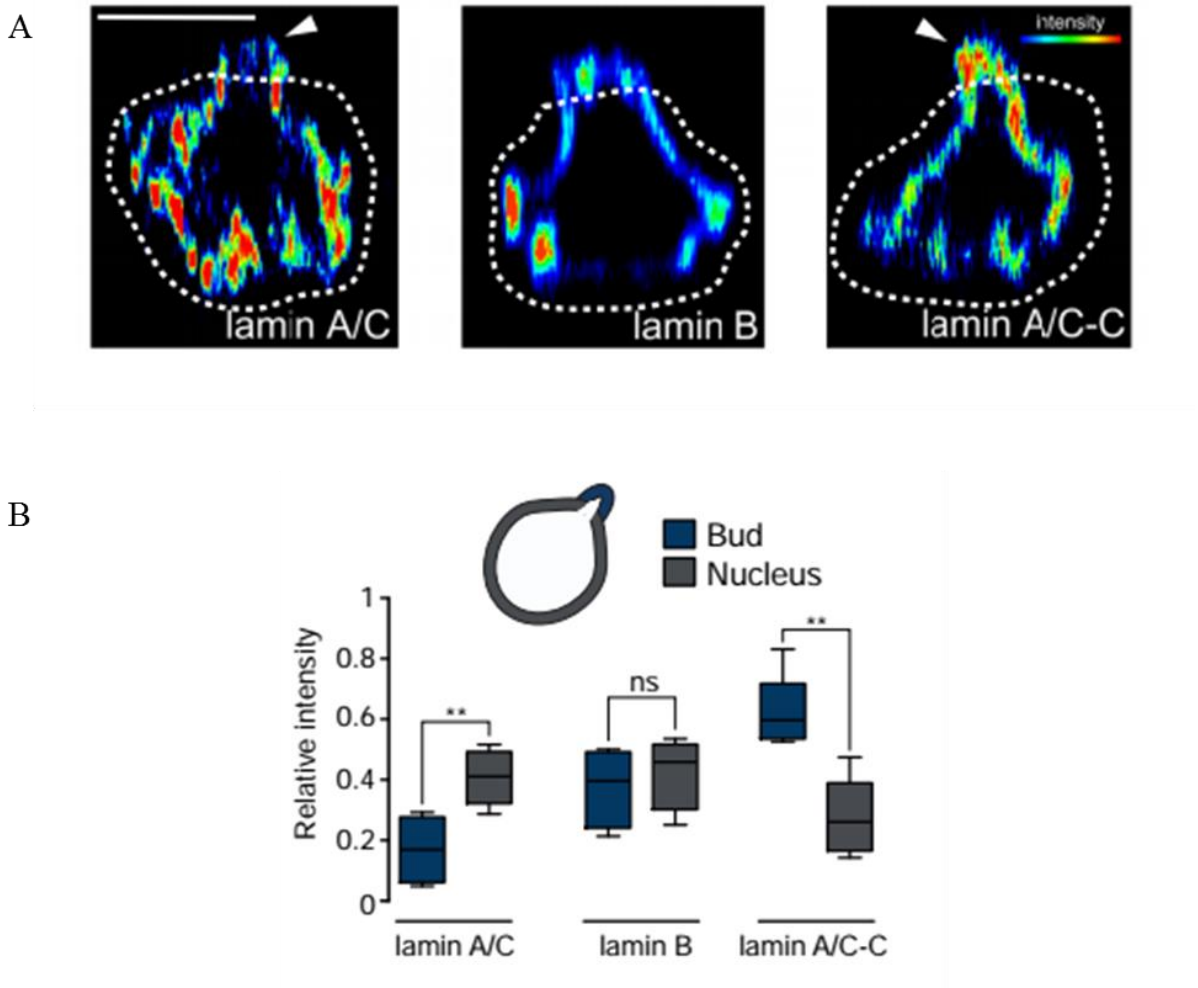


Figure 3.4. Lamin distribution during nuclear squeezing. A Left panel) Orthogonal view of nuclei from 5 day myofibers with bud initiation, stained with Lamin A/C the intensity signal represented as a heat map. White dashed line represents the outline of myofibrils. White arrowheads represent asymmetry nuclear stiffness. A Middle panel) Orthogonal view of nuclei from 5 day myofibers with bud initiation, stained for Lamin B1 with the intensity signal represented as a heat map. White dashed line represents the outline of myofibrils. A Right panel) Orthogonal view of nuclei from 5 day myofibers with bud initiation, stained with Lamin A/C-C the intensity signal represented as a heat map. White dashed line represents the outline of myofibrils. White arrowheads represent asymmetry nuclear stiffness. B) Box plot comparing the intensity of Lamin A/C, Lamin B or Lamin A/C-C in the part of the nucleus still inside the myofibril bundle (grey box plot: nucleus) versus the forming bud (dark blue box plot: bud). (n = 5 for each condition).

To determine if this asymmetrical Lamin A/C distribution was only happening when nuclei started their migration towards the periphery we looked into centrally located nuclei of wild type cells. Even though these nuclei were subjected to pressure they did not show any signs of asymmetrical distribution (Fig 3.5.A). Interestingly, when we observed peripheral nuclei we also found

asymmetrical distributed Lamin A/C (Fig. 3.5.B) This observation could be explained by contact forces exerted on the nucleus in later stages of development.

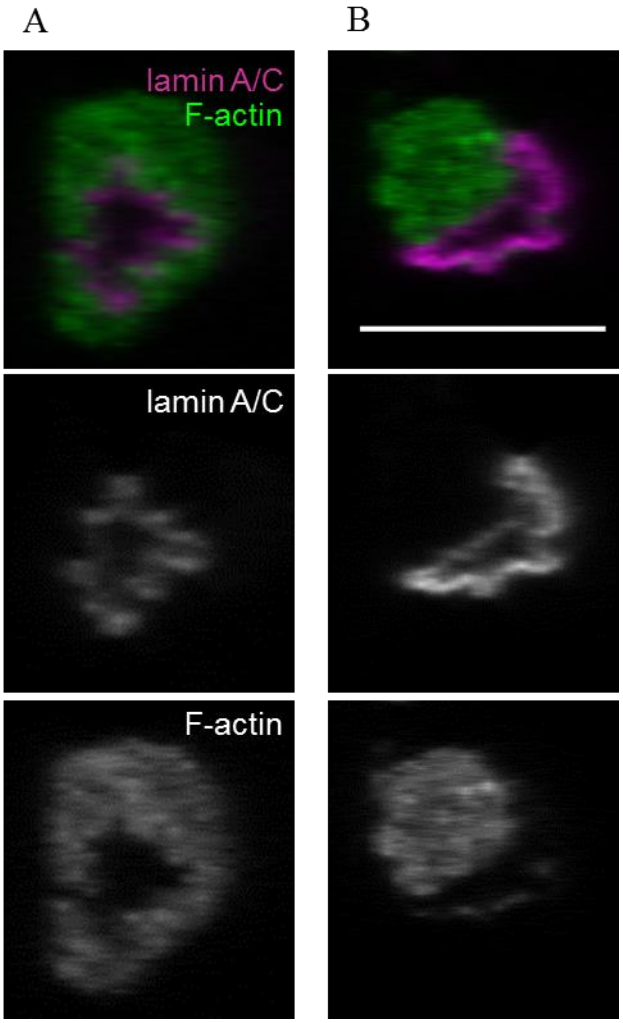


Figure 3.5. Lamin A/C distribution before and after nuclear movement to the periphery. Representative immunofluorescence images of A) 4 day wild type myofibers and B) 10-day wild type myofibers stained for Lamin A/C-C (magenta), F-actin (phalloidin, green).

3.1.3. Lamin A/C dynamics during nuclear movement to the periphery

To further study Lamin A/C distribution and dynamics during nuclear positioning at the periphery we decided to do live imaging of mCherry-Lamin A/C transfected cells at day 5. In order to perform a FRAP experiment it is required to photobleach a region of the sample with a high intensity laser first. The idea behind this experiment was to photobleach only half a nucleus to sharply see Lamin A/C dynamics during nuclear positioning. To do this we closed the field diaphragm to the minimum and

scanned the sample with 100% intensity 555 laser followed by image acquisition in 5 minute intervals (Fig. 3.6.).

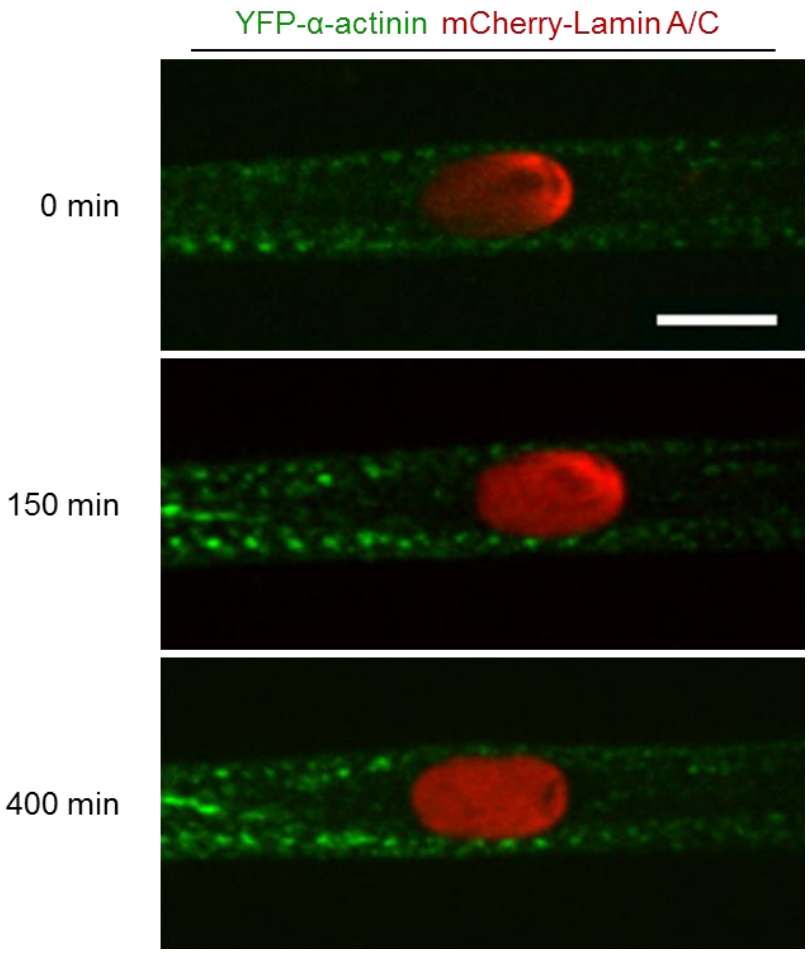


Figure 3.6. Lamin dynamics during nuclear movement. Kymograph from a time-lapse movie of a 5-day myofiber depicting fluorescence recovery of photobleached nucleus during nuclear movement with the nucleus (mCherry-Lamin A/C, red) migrating between myofibrils (YFP-α- actinin, green). Scale bar, 10 μm.

There were a few problems with these experiments. Firstly, the laser was not strong enough even at 100% intensity which made the photobleaching step too time-consuming allowing nuclei to move out of focus. On the other hand, fluorescence recovery occurred to quickly making the observation of Lamin A/C dynamics during nuclear movement to the periphery impossible (Fig. 3.6.). Furthermore, mCherry-Lamin A/C construct might not be functional which indicates that additional experiments are required in order to determine it.

3.2. Myofibril Crosslinking

3.2.1. Analysis of Desmin function in nuclear movement to the periphery

Myofibril crosslinking was predicted to be vital for nuclear movement to the periphery, particularly to orientate the forces applied in the nucleus during its movement. Desmin is known to crosslink myofibrils in muscle cells forming a network that runs through the z-line (Clemen et al., 2013). The initial goal was to determine whether this protein played a role in nuclear movement to the periphery or not. To do this we transfected cells with Desmin siRNA that had previously been tested in the laboratory. Using this method we verified that Desmin depleted cells show a severe decrease in peripheral nuclei, however myofibril structure is not disrupted (Fig. 3.7.B, C). Additionally, we observed that the Desmin network was organized at the Z-lines prior to nuclear movement to the periphery but only in regions away from centrally located nuclei while in region near centrally located nuclei it was disorganized (Fig. 3.7.A). This data suggests that the Desmin network is responsible for proper force transmission through myofibril contraction and thus move nuclei to the periphery, though it seems it does not influence myofibril structural organization. To further study Desmin dynamics during nuclear movement to the periphery, we visualized EmGFP-Desmin transfected cells at day 5 of development by time-lapse epi-fluorescence microscopy in 15 minute intervals. Over time, we were able to observe the formation of a Desmin network in striations originating from the more distal region towards the nucleus, similar to the myofibril zipping observed previously in the laboratory (Fig. 3.7.D). These results further support the hypothesis that Desmin is responsible for myofibril crosslinking and zipping, squeezing the nucleus to the cell periphery.

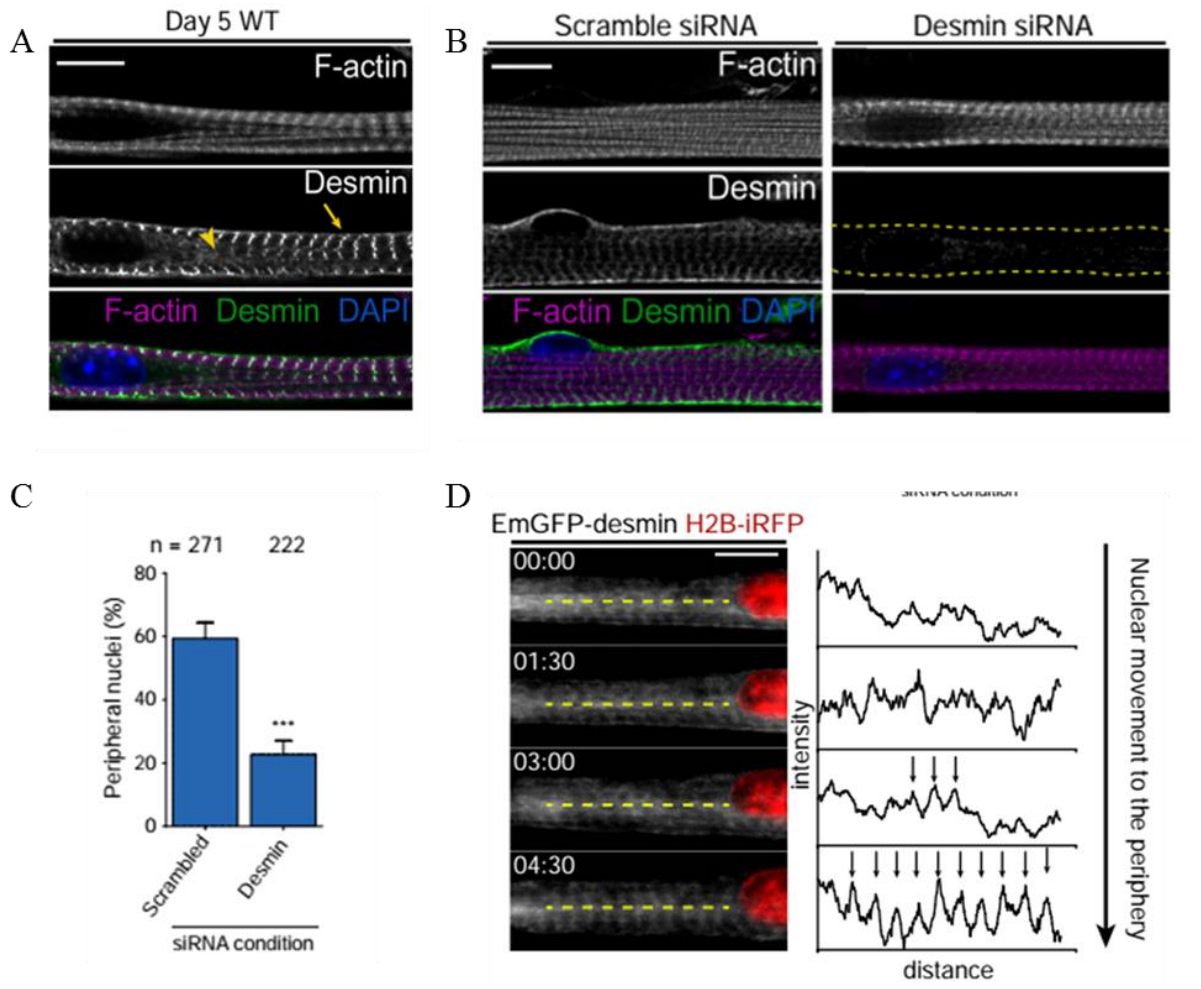


Figure 3.7. Myofibril crosslinking by Desmin drives nuclear movement to the periphery. A) Representative immunofluorescence image of a 4.5-day wild type myofiber stained for F-actin (phalloidin, magenta), Desmin (green) and DAPI (nucleus, blue). Arrow indicates organized Desmin whereas arrowhead indicates disorganized Desmin. Scale bar, 10 μm. B) Representative immunofluorescence image of a 10-day myofiber knocked down for scrambled or Desmin and stained for F-actin (phalloidin, magenta), Desmin (green) and DAPI (nucleus, blue). Scale bar, 10 μm. C) Quantification of peripheral nuclei positioning and traversal triads in 10-day myofibers knocked down for scrambled or Desmin. D) Kymograph from a time-lapse movie of a 5-day myofiber depicting Desmin organization (Emerald-Desmin, gray) during nuclear (H2B-iRFP, red) movement to the periphery. Yellow dashed lines represent the region used to perform line scans plotted on the right. Arrows highlight the transversal organization of Desmin. Time, hh:mm. Scale bar, 10 μm.

3.2.2. Crosslinking organization role in nuclear movement to the periphery

It was previously discovered by our laboratory that nuclear positioning is specifically mediated by Arpc5L-containing Arp2/3 and γ actin, which are colocalized in small patches between myofibrils near

centrally located nuclei at day 5 of development. Moreover, Desmin is the myofibril cross-linker necessary for nuclear movement to the periphery according to our data. Therefore we tested to see if Arpc5L and γ actin were important for Desmin network organization to occur. Depletion of both Arpc5L and γ actin through siRNA mediated knockdown resulted in disorganization of the Desmin network which is no longer localized to the z-lines (Fig. 3.8.A). This suggests that Arpc5L and γ actin are responsible for Desmin organization at the z-lines during nuclear positioning. Desmin network organization presented no changes in Arpc5, β actin and Silencer siRNA control conditions (Fig.3.8.B). This is not unexpected given that previous results indicated that Arpc5 and β actin are not involved in the mechanism of nuclear positioning at cell periphery.

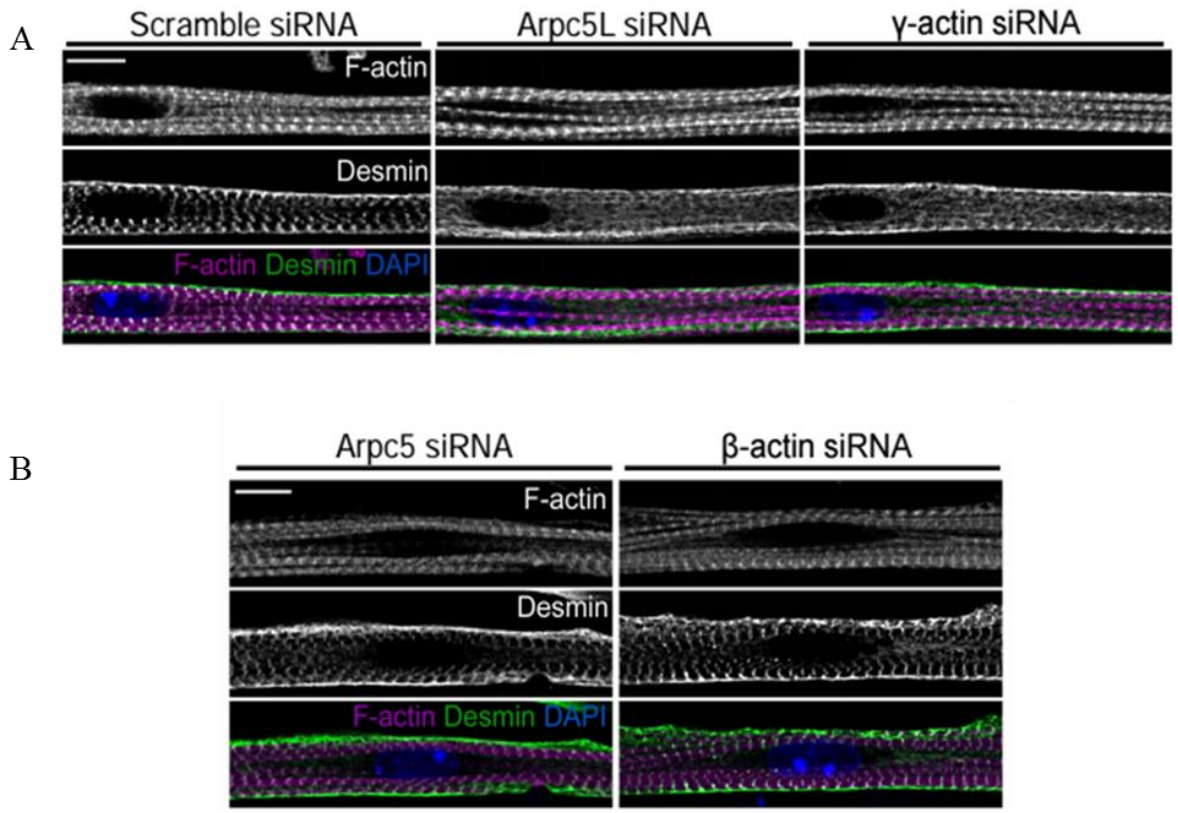


Figure 3.8. Arpc5L and γ actin organize Desmin to cross-link myofibrils for nuclear movement. A) Representative image of 5-day myofibers knocked down for scramble, Arpc5L or γ-actin and stained for F-actin (phalloidin, magenta), Desmin (green) and DAPI (nucleus, blue). Scale bar, 10μm. B) Representative immunofluorescence image of a 4.5-day myofiber knocked down for Arpc5 or β-actin and stained for F-actin (phalloidin, magenta), Desmin (green) and DAPI (nucleus, blue). Scale bar, 10μm.

The cytoskeletal linker, Plectin, was shown to connect Desmin to the z-lines (Konieczny et al., 2008). To determine the role of Plectin in this nuclear positioning mechanism we depleted cells using siRNA mediated knockdown. Initially we tested three different Plectin siRNAs, determined their efficacy and phenotype to choose one to use in the experiments. After these preliminary tests only Plectin 2 siRNA

was used since the levels of knockdown were very similar to the other two but cell survivability was much greater. Knockdown was confirmed by immunofluorescence and Western blotting (Fig. 3.9.A, E Left panel, C). Plectin depleted myofibers presented a significant decrease in peripheral nuclei coupled with Desmin disorganization, worse than in Arpc5L and γ actin depleted cells (Fig 3.9.A, D). Furthermore, when we observed wild type myofibers at day 4, with nuclei still centrally located, Plectin was already organized contrary to Desmin (Fig. 3.9.E Right panel). At day 5, before nuclei moved to the periphery, Plectin was colocalized with Desmin at the z-lines (Fig. 3.9.A Left panel). This data suggests that Plectin is in fact involved in Desmin organization similarly to Arpc5L and γ actin. In order to further understand this mechanism and to try to understand how these proteins interact we tested to see if Arpc5L and γ actin were involved in Plectin organization. Cells depleted of these proteins through siRNA mediated knockdown did not show disruption of Plectin organization (Fig. 3.9.B). This shows that Arpc5L and γ actin act in parallel or downstream of Plectin to organize Desmin for nuclear movement to the periphery.

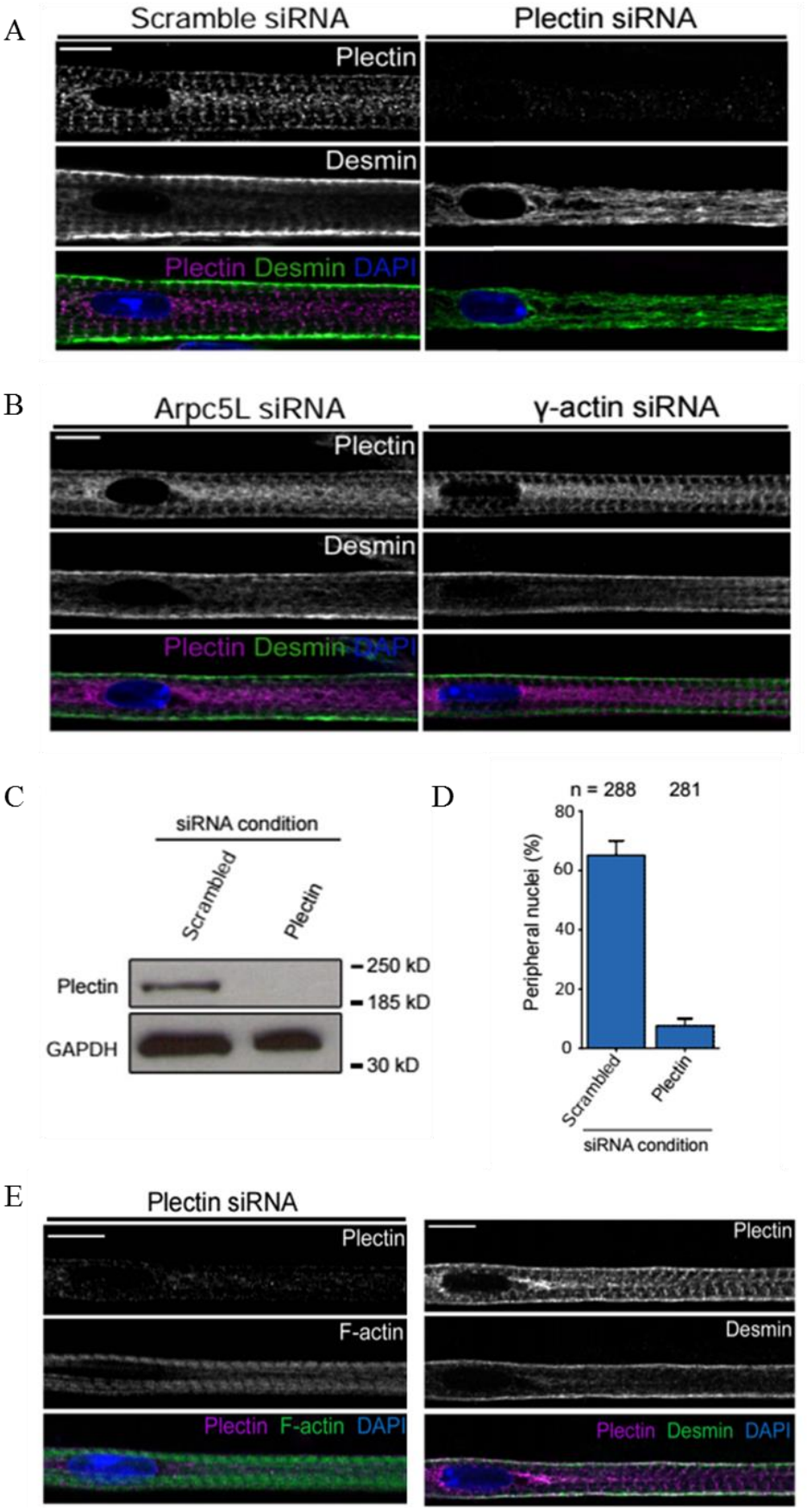
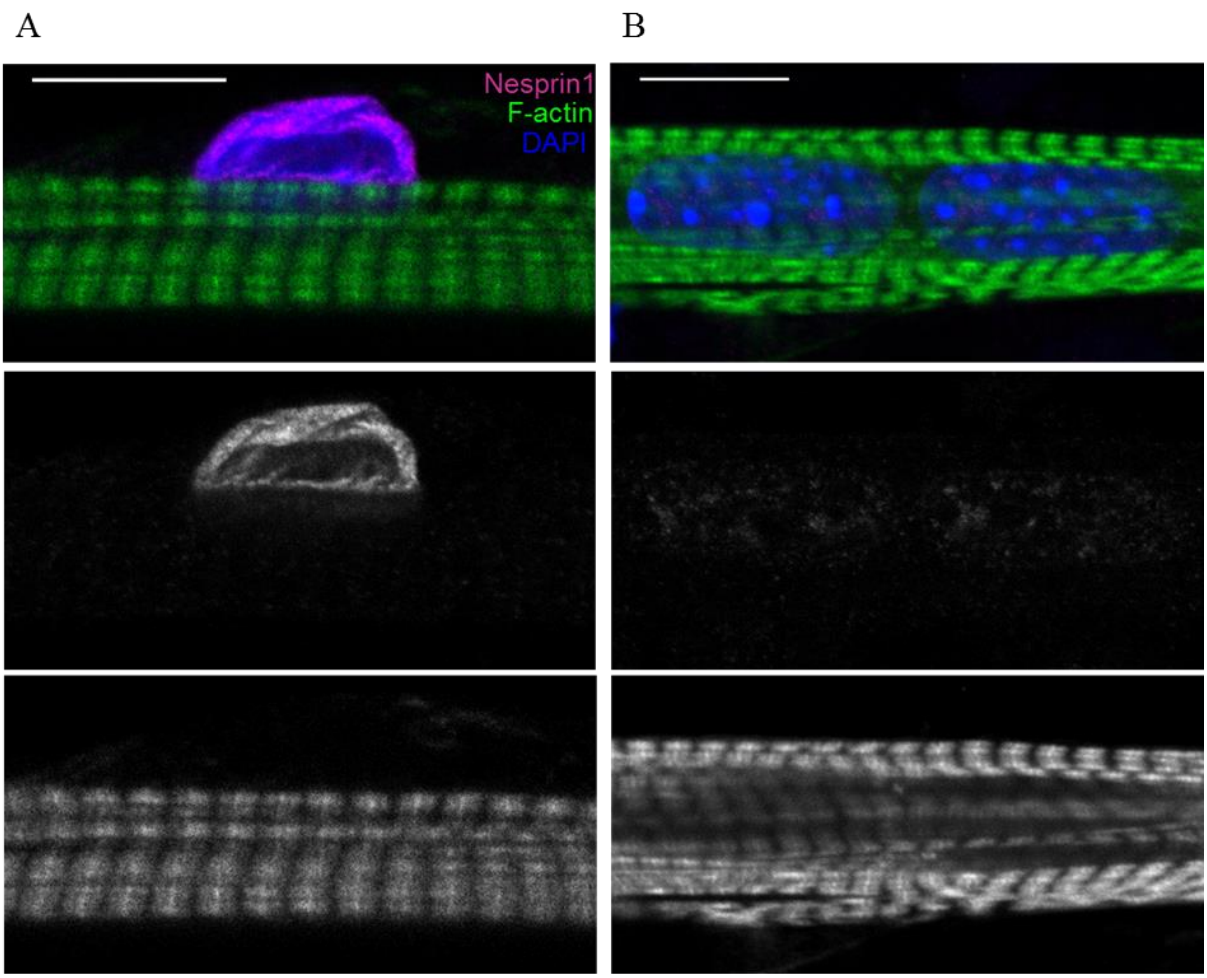


Figure 3.9. Plectin is involved in Desmin organization at the z-lines. A) Representative immunofluorescence image of a 4.5-day myofiber knocked down for scrambled or Plectin and stained

for Plectin (magenta), Desmin (green) and DAPI (nucleus, blue). Scale bar, 10 μm. B) Representative immunofluorescence image of a 4.5-day myofiber knocked down for Arpc5L or γ-actin and stained for Plectin (magenta), Desmin (green) and DAPI (nucleus, blue). Scale bar, 10 μm. C) Western blot with indicated antibodies from 10-day myofibers knocked down for scrambled or Plectin. D) Quantification of peripheral nuclei positioning in 10-day myofibers knocked down for scrambled or Plectin. E Left panel) Representative immunofluorescence image of a 4.5-day myofiber knocked down for Plectin and stained for F-actin (phalloidin, green), Plectin (magenta) and DAPI (nucleus, blue). Scale bar, 10 μm. E Right panel) Representative immunofluorescence image of a 3.5-day myofiber stained for Plectin (magenta), Desmin (green) and DAPI (nucleus, blue). Scale bar, 10 μm.

3.3. Cytoskeleton anchorage to Nucleoskeleton and its influence in nuclear movement to the periphery

KASH domain proteins, Nesprins, are the outer nuclear membrane component of the LINC complex. These proteins are known to be involved in nuclear positioning at the periphery as well as other nuclear movements in myofibers (Falcone et al., 2014). Since Nesprin family is composed by several different isoforms and splicing variants and many are tissue specific, determining which ones play a role in nuclear movement to the periphery was the initial step. For this, wild type cells were fixed at day 10 of development and stained for Nesprin1, Nesprin2 and Nesprin3. All these Nesprin isoforms were reported to be expressed in skeletal muscle although Nesprin2 and Nesprin3 expression seems to diminish greatly or even stop at later stages of cell development, more specifically after fusion. The results showed that Nesprin1 is localized to the nuclear envelope while Nesprin2 and Nesprin3 are scattered throughout the cell. One possible explanation for this would be that the immunofluorescence did not work properly since the signal pattern of Nesprin2 and Nesprin3 staining is very similar to that of antibody unspecific binding. Therefore we initially focused our efforts in trying to determine Nesprin1 function in this mechanism. Nesprin1 depletion was achieved by siRNA mediated knockdown. We observed that Nesprin1 depleted cells presented a significant decrease in peripheral nuclei (Fig. 3.10. A, B, C) similar to the one observed in Lamin A/C depleted cells (Figure 3.2.A, B). Furthermore, nuclei are frequently clustered (Figure 3.10.B) which might be explained by the fact that Nesprin1 is also involved in nuclear spreading (Cadot et al., 2012, Wilson and Holzbaur, 2015). This may indicate that Nesprin1 plays a role in nuclear movement to the periphery.



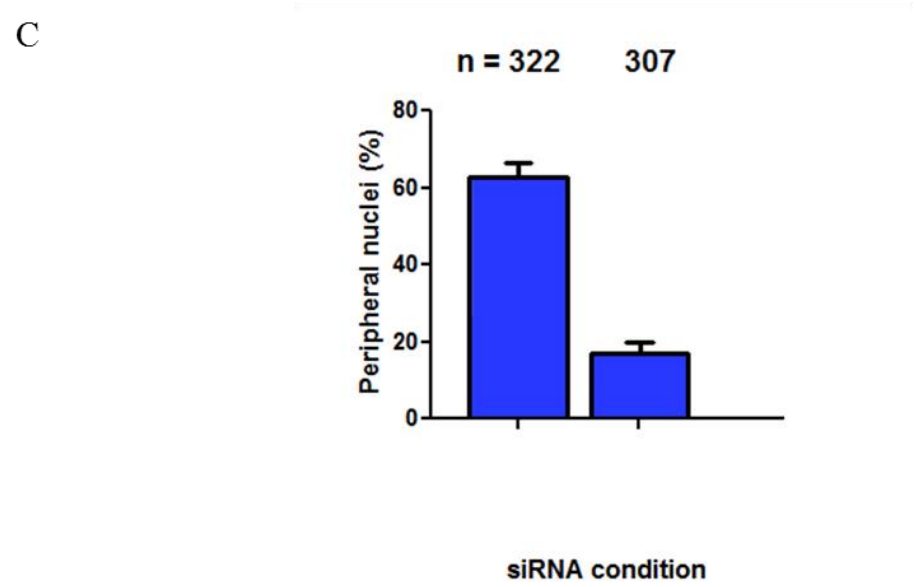


Figure 3.10. Nesprin1 is involved in nuclear movement to the periphery. Representative image of 10-day myofibers knocked down for A) scramble or B) Nesprin1 and stained for Nesprin1 (magenta), F-actin (phalloidin, green), and DAPI (nucleus, blue). Scale bar, 10 μm. C) Quantification of peripheral nuclei positioning in 10-day myofibers knocked down for scrambled or Nesprin1.

Taking our data into account and the fact that LINC complex is responsible for force transmission to the nucleus, we hypothesized that Nesprin1 could present some changes in its distribution throughout the nuclear envelope, in the same way as Lamin A/C. To determine whether or not this was happening we fixed cells at day 5 of development and stained them for Nesprin1. By identifying nuclei squeezing between myofibrils we were able to assess Nesprin1 distribution and our results, although still preliminary, suggest that Nesprin1 does not suffer any alterations of its distribution (Fig. 3.11.).

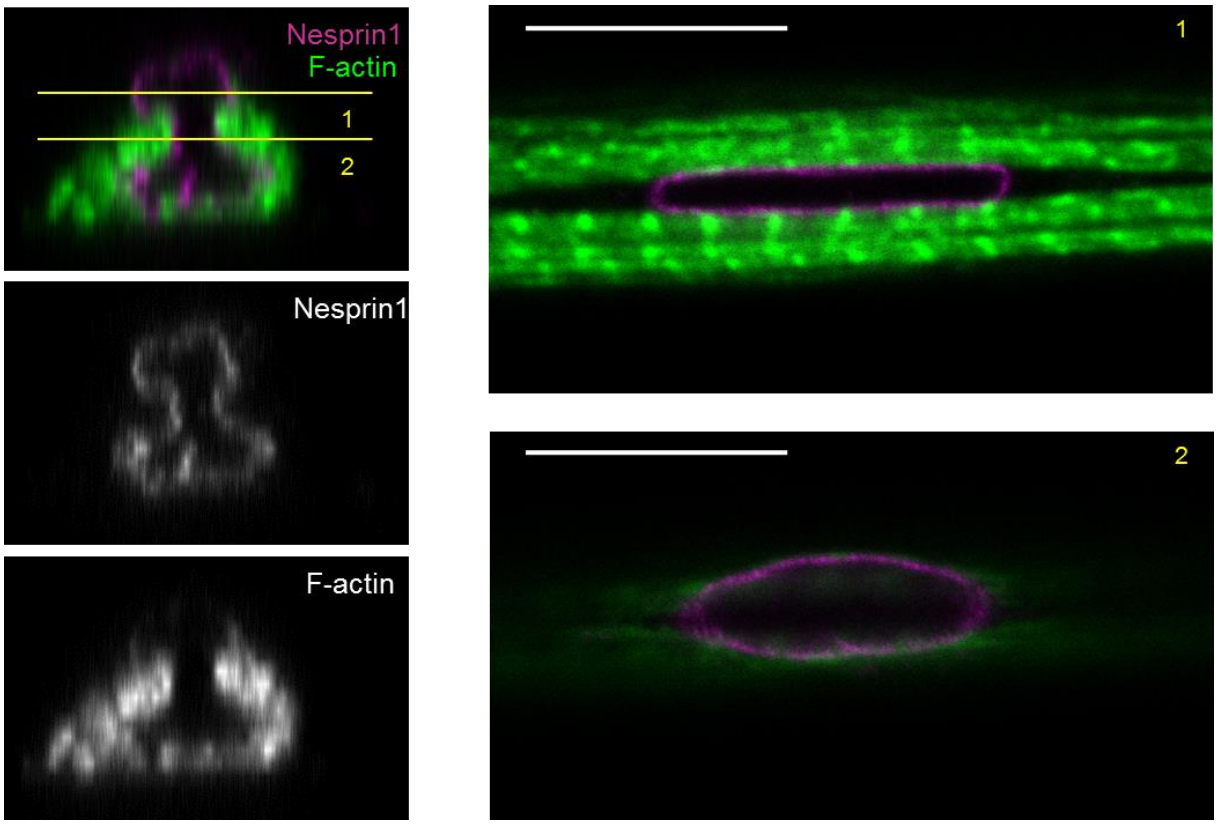


Figure 3.11. Nesprin1 distribution throughout the nuclear envelope during nuclear squeezing. Representative image of a nucleus squeezing to the periphery from an in vitro 5 day myofiber stained for myofibrils (α-actinin, green) and nucleus (Nesprin1, magenta). Left: 2D orthogonal view, yellow lines represent slices seen in right side panels. Top right: 2D plane from yellow slice 1. Bottom right: 2D plane from yellow slice 2. Scale bar, 10 μm.

4. Discussion

4.1. Nuclear stiffness and mechanosignaling

Nuclear positioning mechanisms are essential for a large number of cellular functions, including cell polarization, differentiation and migration. Until recently all described nuclear movements required the polarization of the machinery responsible for exerting force in the direction of movement (Gundersen and Worman, 2013). The nuclear positioning mechanism responsible for nuclear movement to the periphery of myofibers does not require polarized machinery. Instead, the forces induced by myofibrils are centripetal and symmetrically distributed, with bud formation being pivotal for the polarization of the movement (Roman et al., 2016 under revision). Bud formation is dependent on local changes in nuclear stiffness regulated by nuclear lamina, in particular Lamin A/C. In fact, a drastic decrease in peripheral nuclei was observed upon Lamin A/C depletion along with elongated nuclei and nuclear breaks allowing nucleoplasm to escape the nucleus. Nuclei without or with low levels of Lamin A/C are very fragile and susceptible to the forces applied by myofibrils resulting in their inability to migrate to the periphery and nuclear ruptures. The elongated nuclear shape and ruptures are consistent with low Lamin A/C levels characteristic of laminopathies and cancer, especially when cells are subjected to mechanical pressure (De Vos et al., 2011; Raab et al., 2016; Vargas et al., 2012).

Conversely, Lamin A/C overexpression resulted in an increase of nuclear stiffness with myofibrils not being able to exert enough pressure to squeeze nuclei to the periphery. Even though, a few nuclei are still able to migrate to the periphery, probably due to lower levels of Lamin A/C overexpression. These nuclei move much slower than nuclei with endogenous levels of Lamin A/C (Roman et al., 2016 under revision). Our data suggests that changes in nuclear stiffness, whether it is a decrease or an increase, hinder nuclear movement to the periphery of myofibers. However Lamin A/C constructs such as mCherry-Lamin A/C might not be functional since the addition of the extra sequence could impede the assembly of the Lamin A/C meshwork. Therefore further analysis is required, more precisely through rescue experiments which could be achieved by knocking down Lamin A/C and trying to rescue the phenotype with mCherry-Lamin A/C transfection.

Recent work demonstrated that in migrating cells, the nuclear envelope can rupture and be repaired when forced to pass through confined spaces (Denais et al., 2016; Raab et al., 2016). Our data does not provide any evidence indicating a similar event occurs during nuclear movement to the periphery since we do not observe absence of Lamins in the nuclear envelope or such severe nuclear deformations.

Nuclear stiffness plays an important role in nuclear movement to the periphery, displaying local variations throughout the nuclear lamina essential for bud formation and nuclear squeezing. Our data

shows that only Lamin A/C is asymmetrically distributed when nuclei are squeezed by myofibrils. The area being squeezed displays an increase of Lamin A/C levels in order to resist myofibril forces while the bud displays a decrease of Lamin A/C levels facilitating movement towards the periphery of the cell. The Lamin A/C-C antibody which is epitope specific allowed the detection of a Lamin A/C multimerization reduction in the bud consistent with what was observed previously. This suggests that Lamin dynamics change during this process in response to certain cues, the most likely being mechanical stress. Centrally located nuclei of 4 day myofibers do not display Lamin A/C asymmetry even though pressure is being applied by myofibrils. A possible explanation for this would be that the force exerted by myofibrils is still insufficient to trigger Lamin A/C asymmetry since Desmin crosslinking network is still not formed. Interestingly enough, peripheral nuclei also present an asymmetrical distribution of Lamin with Lamin A/C levels increasing in the area that contacts the myofibrils. This could be a way to protect the nucleus from contraction. Lamina coupling with the cytoskeleton through LINC complex allows force transmission from the extracellular matrix and possibly from myofibril zipping and contraction (Lombardi et al., 2011). A study indicates that force transmission regulates Lamin levels and therefore nuclear stiffness (Swift et al., 2013). This suggests an outside-to-inside mechanosignaling, with myofibrils transmitting forces to the nucleus causing deformations in the nuclear envelope which in turn will alter chromatin organization and transcription factor accessibility. One hypothesis in myofibers is that force transmission during nuclear squeezing might trigger a change in expression patterns allowing proper cell development, with nuclear envelope stiffness playing a crucial role.

Our attempts to study Lamin A/C dynamics during nuclear movement by FRAP did not yield the expected results since Lamin A/C presented a high protein turnover resulting in fast fluorescence recovery. Future work should address if the local changes in Lamin distribution are triggered by mechanotransduction or by another mechanism in addition to determining Lamin dynamics during nuclear squeezing. This could be achieved by using forster resonance energy transfer (FRET)-based Molecular Tension microscopy, which allows mechanical force measurements when using Nesprin or SUN tension sensor constructs (Gayrard and Borghi, 2016). Coupling it with Lamin A/C overexpression would allow us to correlate force intensity with Lamin A/C levels although it will also hinder nuclear movement to the periphery.

Lamins interact with a large number of proteins that might also play a role in nuclear movement to the periphery. If mechanosignaling from myofibrils to the nucleus culminates in an alteration of gene expression then LEM domain proteins are likely involved since they are known to bind DNA and even influence chromatin organization (Cai et al., 2001). Moreover loss of Emerin causes X-Linked Emery-Dreifuss muscular dystrophy which presents some similarities with CNM (Bione et al., 1994). Despite most being transmembrane proteins, LAP2α is localized to the nucleoplasm where it interacts with A-type Lamins also affecting chromatin organization (Zhang et al., 2013). It is possible that, upon force

transmission and lamina deformation, the signaling cascade alters interactions between LEM domain proteins and DNA, thus chromatin organization changes. Another possibility encompasses transmembrane LEM domain proteins asymmetrical distribution throughout the nuclear envelope, similar to Lamin A/C asymmetry described in this work. In this case, asymmetrical distribution of LEM domain proteins caused by forces exerted by myofibril would originate a severe change in chromatin organization near the edge of the nucleoplasm. The distribution of LEM domain proteins during nuclear movement to the periphery would probably be very similar to the distribution of Lamin A/C, since there is inter-dependence between lamin, some LEM domain proteins and BAF (Liu et al., 2000; Margalit et al., 2005). In addition to these hypotheses, Lamin is known to bind BAF directly and consequently DNA. It is possible that alterations of chromatin organization would result from Lamin A/C interaction with BAF during nuclear movement to the periphery without any LEM domain protein involvement.

A potential way to approach and test this hypothesis would be to deplete cells of several LEM domain proteins and BAF in order to determine the phenotype and in particular determine Lamin A/C distribution under these conditions. Since LEM domain proteins are partially redundant a phenotype might not be clear when knocking down each one of them individually. Furthermore, time lapse analysis of BAF dynamics during nuclear movement to the periphery would allow us to determine if, similarly to pre-Lamin A nuclear localization, BAF is required for Lamin A/C localization to high tension areas (Loi et al., 2016).

4.2. Myofibril crosslinking

During nuclear movement to the periphery a Desmin network is formed at the z-lines towards centrally located nuclei, crosslinking myofibril and zipping them around the nucleus leading to movement to the periphery. Our data suggests that Plectin works side by side with Arpc5L and γ actin to organize the Desmin network. This whole process might use similar mechanisms found in migrating cells where actin retrograde flow organizes the vimentin IF cytoskeleton via Plectin (Jiu et al., 2015).

A recent study showed that Desmin IF network is anchored to the outer membrane of nuclei by Plectin1 isoform indicating that myofibril crosslinking and zipping might be involved in mechanosensing pathways during nuclear movement to the periphery. In addition, Desmin could be responsible for other cellular functions prior to nuclear movement that would influence proper cell development. In our experiments, transfections are performed at day 0 of development, 5 days before nuclear movement to the periphery. During this period other Desmin dependent cellular processes would be affected, influencing the results obtained from Desmin depletion assays. To exclude this possibility it would be ideal to perform the knockdown at day 4 of development, however this would

have to be done by infection with a viral vector since in our model transfection is only possible at day 0 of development. Furthermore, Plectin depletion was directed at all Plectin isoforms which might affect other Plectin dependent cellular mechanisms. Future work should focus on studying the role of Plectin isoforms separately, more specifically Plectin1 and Plectin1d isoforms that anchor Desmin to the nucleus and the z-lines respectively.

4.3. LINC complex role in mechanosignaling

With a broad range of function such as nuclear movement, signal transduction and chromosome movement, the LINC complex might play a pivotal role in nuclear movement to the periphery. To explore this we first determined which Nesprin isoforms where expressed in skeletal muscle and what was their function in nuclear movement by siRNA mediated knockdown. Nesprin1 was localized to the nucleus while Nesprin2 and Nesprin3 showed low levels of expression at day 5 of development, thus we focused on Nesprin1 function. Our data shows a severe decrease of peripheral nuclei in Nesprin1 depleted cells and nuclear clustering which was to be expected since Nesprin1 is involved in nuclear movement mechanisms prior to migration to the periphery (Falcone et al., 2014). Furthermore Nesprin1 does not display asymmetrical distribution during nuclear squeezing although the small sample size does not allow for statistical analysis and so results remain preliminary. These results suggest a role in nuclear movement to the periphery even though further work is required to determine if the LINC complex is a part of a mechanosignaling pathway essential for myofiber development.

Firstly, it would be important to determine LINC complex interactions and anchorage at the nuclear lamina. The LINC complex requires proper anchorage at the nuclear envelope so that it is capable of transmitting forces to the nucleus resulting in nuclear movement. Lamins are known to contribute to this anchoring, mainly through Lamin A binding to SUN proteins since Lamin B and C present a very weak binding (Crisp et al., 2006). Moreover SUN proteins show increased diffusional mobility in cells devoid of Lamin A or with low levels (Ostlund et al., 2009). In some cases, LINC complex mediated functions still occur even in the absence of Lamins suggesting the involvement of other proteins in its anchorage (Kim et al., 2011). Lamin associated proteins such as Emerin may also contribute to LINC complex anchoring since Emerin depletion in polarizing fibroblasts leads to abnormal migration (Chang et al., 2013). This suggests that LINC complex could affect chromatin organization to a certain extent via Lamin A binding or Emerin binding. Possibly, LINC complex anchorage at the nuclear lamina is altered due to force transmission from myofibrils and this determines local alterations in chromatin organization.

4.4. Nuclear positioning in muscle disorders

Some of the genes encoding the proteins involved in the described mechanism are mutated in different muscle disorders, most of which exhibit centrally located nuclei. *BIN1*, which encodes for Amphiphysin-2 is mutated in centronuclear myopathies and miss-spliced in myotonic dystrophy (Fugier et al., 2011; Nicot et al., 2007). Amphiphysin-2 mutations disrupt N-Wasp localization and activity thereby probably preventing Arp2/3-dependent nucleation of γ-actin for Desmin cross-linking (Falcone et al., 2014). Furthermore, Emery-Dreifuss muscular dystrophy is caused by mutations in the *LMNA* gene (Azibani et al., 2014). *LMNA* mutations associated with muscular dystrophy were found to cause a reduction in nuclear stiffness (Lammerding et al., 2004) . The inability to regulate nuclear stiffness in these disorders probably hinders nuclear movement to the periphery of myofibers similarly to what we have shown here. Lamin A/C deficiency also affects the expression of proteins essential for proper muscle differentiation such as Desmin (Columbaro et al., 2005). Emery-Dreifuss muscular dystrophy can also be caused by mutations in Nesprin genes affecting both nuclear spreading and nuclear movement to the periphery (Zhang et al., 2007). Finally, desminopathies and plectinopathies, which result from mutations in the Desmin and Plectin genes respectively, also exhibit misplaced nuclei in addition to Desmin aggregates (Clemen et al., 2013). Desmin and Plectin mutations are known to cause myofibril crosslinking defects.

Our observations on the mechanism of nuclear positioning demonstrate why mutations in diverse proteins lead to a common phenotype of centrally located nuclei in different muscle disorders. However, it remains unclear whether centrally located nuclei are just a part of the phenotype displayed in several muscle disorders or if the fact they are centrally located gives rise to some of the phenotypical features. To shed some light on this subject it would be necessary to determine the function of LEM domain proteins and LINC complex followed by analysis of their interactions with cytoskeleton, Lamins and chromatin. Moreover, it would be required to identify possible changes in chromatin organization and finally study alterations in gene expression.

4.5. Conclusions

Nuclear movement to the periphery of myofibers is crucial for proper muscle function, with several muscular disorders, such as centronuclear myopathies, being characterized by centrally located nuclei. During this movement, nuclei are squeezed between myofibrils and suffer dramatic deformations until they are expelled to the periphery (Roman et al., 2016 under revision). Thus we hypothesized that nuclear stiffness and myofibril crosslinking could play a part in nuclear positioning at the periphery of myofibers.

We demonstrated that Lamin A/C depletion severely hinders nuclear movement to the periphery due to loss of nuclear stiffness. Conversely, overexpression of Lamin A/C resulted in an increase of nuclear stiffness which also affected nuclear positioning. Furthermore, we observed that Lamin A/C is asymmetrically distributed during nuclear squeezing by myofibrils. The bud presented lower levels of Lamin A/C while the region being squeezed by myofibrils presented higher levels when compared to the rest of the nuclear envelope. This suggests that global and local changes in nuclear stiffness determine whether a nucleus is capable of migrating to the periphery or not.

We found that myofibril crosslinking and zipping are Desmin dependent processes, requiring the organization of a Desmin network at the z-lines during nuclear migration to the periphery. Plectin depletion phenotype is similar to that observed in Arpc5L and γ actin depleted cells. Under these conditions Desmin organization at the z-lines and nuclear movement are impaired. Since Desmin depletion does not affect Plectin this indicates that Plectin, along with Arpc5L and γ actin regulate Desmin crosslinking. Additionally, we verified that Nesprin1 isoform influences nuclear movement to the periphery although its specific function in this mechanism remains unknown.

With this work we show that nuclear movement to the periphery of a myofiber is driven by myofibril crosslinking and zipping by a Desmin network which is regulated by Plectin, Arpc5L and γ actin. Moreover it requires tight regulation of nuclear stiffness by Lamin A/C.

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